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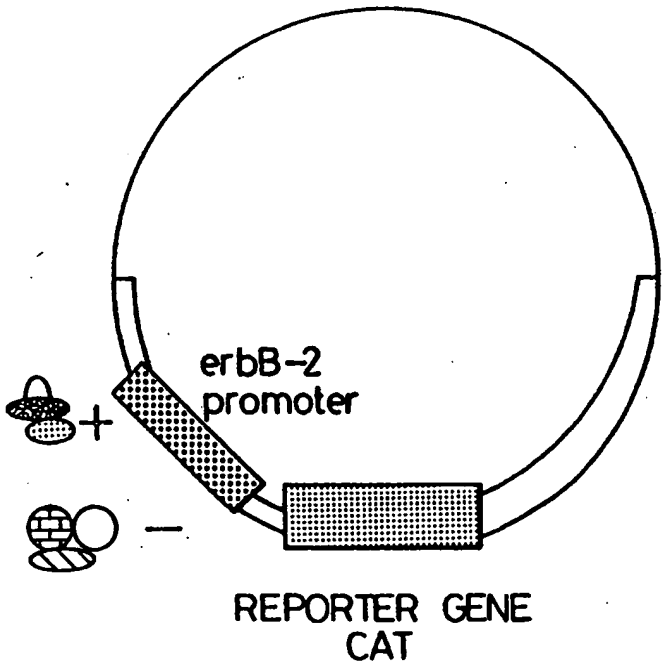
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(57) Abstract

The OB2-1 transcription activator, antibodies reactive thereto and genes encoding OB2-1 are described. Compounds interfering with the binding of OB2-1 to its binding site. Measurement of the abundance OB2-1 in tumours may be used as a prognostic marker and to indicate which tumours are suitable for treatment with the DNA construct and compounds of the invention. A DNA construct comprising (i) a promoter segment which specifically binds the c-*erb*B-2-binding nuclear transcription activator and, under transcriptional regulation thereby, (ii) a heterologous coding sequence. The construct is introduced into tumour cells, where the promoter provides for tumour-specific expression of the heterologous coding sequence, which encodes a directly or indirectly toxic substance, such as cytosine deaminase (CD). CD converts the pro-drug 5-fluorocytosine into the more toxic 5-fluorouracil.



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CANCER TREATMENT

Background and Prior Art

The present invention relates to the treatment of cancer by means of the delivery to tumour cells of DNA constructs which encode a directly or indirectly cytotoxic compound. In this art, selective destruction of the tumour cells, rather than normal cells, may be achieved (at least in theory) by generation of a compound which is more toxic in tumour cells than in normal cells (for example all conventional anticancer drugs), by selective delivery of a cytotoxic compound (for example using tumour-specific antibodies or tumour-specific liposomes) or by selective expression of a cytotoxic compound in the tumour cells (using tumour-cell-specific regulatory elements).

In this art, it is important to identify those cells which are able to selectively express a cytotoxic compound. It is an object of the present invention to identify those cells and tumours which are particularly suited to overexpress genes driven by the c-*erb*B-2 promoter. It is a further object of the invention to identify a factor responsible for tumour-specific expression of c-*erb*B-2 and to identify compounds which inhibit the said factor and thus expression of c-*erb*B-2. It is a still further object of the invention to provide DNA constructs suitable for the expression of a cytotoxic compound in tumour cells, particularly breast tumour cells.

Summary of the Invention

A first aspect of the invention provides the c-*erb*B-2 promoter-binding nuclear transcription activator OB2-1.

A s cond aspect provides a polypeptide immunologically reactive with OB2-1.

The immunologically reactive polypeptides include antibodies which react with OB2-1 and fragments and derivatives of such antibodies.

A third aspect provides nucleic acids encoding OB2-1.

A fourth aspect provides a method to aid prognosis or diagnosis for a cancer patient or to determine their suitability for treatment comprising the steps (1) removing a sample of tumour tissue from the patient, (2) measuring the amount of OB2-1 or AP-2 present in the sample, (3) measuring the amount of a marker compound in the sample, (4) computing a ratio of OB2-1 or AP-2 to marker compound, and (5) comparing the said ratio with a ratio computed in the same manner for non-tumour tissue.

Fifth and sixth aspects provide an assay for a compound which reduces the binding of OB2-1 to its DNA binding site, the assay comprising the steps (1) adding the compound to purified OB2-1; (2) further adding an OB2-1 binding site; and (3) determining whether OB2-1 is bound to the OB2-1 binding site and an assay for a compound which reduces the activity of OB2-1, the assay comprising the steps (1) transfecting a cell with an active promoter comprising an OB2-1 binding site which promoter drives the expression of a reporter gene; (2) adding to the said cell or a product of the said cell the compound; and (3) determining whether the activity of the said promoter is reduced.

By "reduces the activity" of OB2-1 we mean that the compound reduces the ability of OB2-1 to transactivate expression of the reporter gene. Such a reduction may be achieved by the compound reducing the binding of OB2-1 to its DNA binding site or by modifying the productive interaction of OB2-1 with RNA polymerase or by other means.

A seventh aspect of the present invention pr vides a DNA construct comprising (i) a promoter segment which specifically binds the c-*erb*B-2-binding nuclear transcription activator and, under transcriptional regulation thereby, (ii) a heterologous coding sequence.

An eighth aspect provides a vector comprising the said construct and means to introduce the construct into a mammalian cell.

A ninth aspect provides a method of combatting tumour cells, the method comprising the step of introducing the said construct into the tumour cells.

A tenth aspect provides a method of combatting breast cancer, the method comprising the step of administering a gold-containing compound to the patient.

An eleventh aspect provides a method of treating cancer in a patient comprising (1) determining their suitability for treatment using the methods defined in the fourth aspect of the invention and, if the patients are suitable for treatment, (2) administering a tumour-inhibitory amount of a construct as defined in the seventh aspect or compounds as defined in the tenth aspect or compounds identified using the assays of the fifth and sixth aspects of the invention.

Detailed Description of Preferred Embodiments

The invention will now be illustrated by reference to the following non-limiting examples and figures.

Figure 1 shows the c-*erb*B-2/CAT construct of Example 1, with the circles and ovals representing putative transcription regulatory factors.

Figure 2 shows diagrammatically the promoter deletions studied in Example 2.

Figure 3 shows the results of a comparison of activity of the construct of Example 1 in two cell lines: T47D, which is a breast carcinoma cell line with base line *erb*B-2 expression, and ZR75-1, which is a breast carcinoma cell line with elevated *erb*B-2 expression.

Figure 4 shows the pCD/*erb*B2 Neo construct forming part of a retroviral vector (Example 3).

Figure 5 shows the nucleotide sequences of primers CDA4R1 (SEQ ID No. 20) and CDA5AS (SEQ ID No. 21), the corresponding amino acid sequences and the relative positions of these primers in the yeast CDase amino acid sequence CDA4R1 is oriented 5′ to 3′ on the sense strand while CDA5AS is oriented 5′ to 3′ on the antisense strand (Example 7).

Figure 6 depicts the nucleotide sequence of DNA derived from genomic clones encoding yeast CDase (SEQ ID No. 22).

Figure 7 shows the nucleotide sequence and corresponding amino acid sequence of yeast cytosine deaminase cDNA (SEQ ID No. 23).

Figure 8 shows the gross structure of the plasmid pFRSV-c-*erb*B-2.

Figure 9 shows diagrammatically the interaction of the plasmid DNA with the ligand-polycation conjugate resulting in the DNA condensing into compact ligand-coated particles (donuts). The ligand moiety of this donut binds to receptors on the cell surface, allowing the internalisation of the complex into endosomes by receptor mediated endocytosis. The transferred DNA in the endosome is then targetted for lysosomal degradation or, alternatively it may escape from the endosome to reach the nucleus where gene expression can be effected.

Figure 10 shows the nucleotide sequence (SEQ ID No. 24), and deduced amino acid sequence (SEQ ID No. 25), of *E. coli* CDase.

Figure 11 shows the results of CAT assays using heterologous promoter (GST-π) constructs. Short-term transfection assays into ZR75-1 and T47D cells were performed. The CAT activity for the pπ213(3)CAT plasmid is expressed relative to the activity of the pSS0.2CAT plasmid, which achieved ~40% of the activity of pSV2CAT in each of the cell lines transfected.

Figure 12 shows the 5′ flanking sequence with 71 bp of transcribed sequence of the human MUC1 gene (SEQ ID No. 26). The TATA box (boxed) and transcriptional start site (+1) are indicated. The sequence (-787 to +71) covers the region required for maximum transcription of the reporter gene (-743 to +33).

Figure 13 shows regulatory elements within the 5′ flanking sequence of the MUC1 gene promoter.

Figure 14 shows a purification scheme for OB2-1.

Figure 15 shows the DNA sequence of the human c-*erb*B-2 5′ region as determined by Hudson *et al* (1990) *J. Biol. Chem.* **265**, 4389-4393) (SEQ ID No. 27).

Figure 16 shows the DNA sequence of the human c-*erb*B-3 5′ region (SEQ ID No. 28) and the predicted amino acid sequence of the first exon (SEQ ID No. 29).

Figure 17 shows the effect of aurothiomalate on the binding of OB2-1 to its binding site in the c-*erb*B-2 promoter.

Figure 18 shows the effect of aurothiomalate on the expression of chloramphenicol acetyl transferase from the c-*erb*B-2 promoter containing the OB2-1 binding site in MDA MB 453 cells.

Figure 19 shows a diagrammatic representation of the differences between AP-2 and AP-2B. The unshaded bar represents identical sequences between the two proteins whereas the hatched and stippled regions are different.

Figure 20 shows diagrammatically a reporter gene construct suitable for use in an assay of the invention.

The c-*erb*B-2 gene and promoter have been characterised previously and the gene product has been shown to be over-expressed in tumour cell lines (Kraus *et al* (1987) *EMBO J* **6**, 605-610) but this could have been due to loss of a repressor-binding site in the promoter or to increased half-life of the mRNA, neither of which causes would have suggested the use of the wild-type promoter in the constructs of the invention.

We have further characterised the promoter and identified fragments thereof which (i) retain the useful properties of the entire promoter, (ii) can be handled more readily and (iii) may be more specific as a result of the absence of non-specific binding sequences. Thus, such fragments may also result in enhanced differential expression of the associated coding sequence.

A particularly useful fragment comprises all or part of the following sequence (SEQ ID No. 1) (shown here in double-stranded form):

```
-226 GAGAACGGCTGCAGGCAACCCAGGCGT -200
     CTCTTGCCGACGTCCGTTGGGTCCGCA
```

This fragment, or functional parts of it, may be incorporated as tandem repeats of 2, 3, 4, 5 or more, with or without short (up to 100 bases) segment of non-interfering DNA sequence, upstream of the TATA box. The number of repeats and the distance from the TATA box may be optimised by CAT assays in low and high expressing cells.

An example of a useful fragment of the c-*erb*B-2 promoter is GGCTGCAGGC (SEQ ID No. 2). A slightly modified version consists of the sequence GCCTGCAGGC (SEQ ID No. 3). A further fragment of the promoter consists of the sequence CTGCAGG.

The c-*erb*B-2 promoter has been found to have the advantage of directing specific over-expression in tumours, whereas, with other genes which are known to be over-expressed in tumours, either the over-expression is not specific to tumours (for example the EGF receptor gene is over-expressed in many tumours but also in many inflammatory or reactive conditions) or it is due to deletion of a repressor-binding site or to gene amplification (for example n-myc).

Similarly, the c-*erb*B-3 promoter, whose sequence is shown in Figure 16, may be used to make a construct of the invention. In particular, the sequence GCCTCTGGC (SEQ ID No. 4) shown as position 55-63 in Figure 16, and nucleic acids comprising this sequence are useful in making a DNA construct of the invention.

It is preferred if the promoter segment that binds the c-*erb*B-2 nuclear transcription factor are within 200 bp upstream of the transcriptional initiation site.

We have found that when one or more of the sequences SEQ ID No. 1 (or

other pr moter segments of the invention which specifically bind the c-*erb*B-2-binding nuclear transcription activator such as SEQ ID No. 4) are introduced into a heterologous promoter region ie one in which it does not normally occur, then this resulting promoter is useful in the practice of the present invention as it directs selective over-expression in tumours. Such an example of a heterologous promoter region is that from the GSTπ gene as described in the examples. Further selectivity of the promoter is conferred by including such sequences into a promoter that is specific for epithelial or mammary cells.

Thus, in a preferred embodiment the promoter segment of the DNA construct additionally comprises a segment from an epithelial cell specific promoter or from a mammary cell specific promoter, the segment being one which confers cell-type selectivity.

A suitable epithelial cell specific promoter is the MUC1 promoter described in detail in WO 91/09867 incorporated herein by reference; a suitable mammary cell specific promoter is the MMTV LTR promoter described by Mink *et al* (1992) *Mol. Cell. Biol.* 12, 4906-4918 incorporated herein by reference.

Sequences within the MUC1 promoter and the MMTV LTR promoter, and not necessarily the whole promoter region, are known to confer tissue selectivity. Thus, it will be appreciated that the minimal region of each promoter which confers tissue selectivity, either in single or multiple copy, may be combined with one or more promoter segments of the present invention to provide a promoter with enhanced selectivity for epithelial cancer cells, in particular breast cancer cells. Clearly, the selectivity-conferring regions of the epithelial cell or mammary cell specific promoters may be placed upstream or downstream of the promoter segment of the invention; if more than one of each region or promoter segment are present, they may be interspersed with each other.

The 743 bp of 5' flanking sequence of MUC1, immediately upstream of the transcriptional initiation site, is preferred.

It is preferred if the region of the MMTV LTR promoter comprises the mammary cell-activating factor (MAF) binding site which has a consensus sequence G Pu Pu G C/G A A G G/T (SEQ ID No. 5). It is further preferred if multiple copies of the MAF binding site are present in conjunction with the nuclear transcription activator binding site in the DNA construct of the invention.

The "heterologous coding sequence" is heterologous to the promoter. By "directly or indirectly" cytotoxic, we mean that the product expressed by the coding sequence may itself be toxic (for example tumour necrosis factor) or it may be metabolised to form a toxic product, or it may act on something else to form a toxic product. The latter process is preferred, and we particularly prefer to express an enzyme which converts a pro-drug to a toxic drug. The enzyme cytosine deaminase converts 5-fluorocytosine (5FC) to 5-fluorouracil (5FU) (Mullen *et al* (1992) *PNAS* 89, 33); the herpes simplex enzyme thymidine kinase sensitises cells to treatment with the antiviral agent ganciclovir (GCV) (Moolten (1986) *Cancer Res.* 46, 5276; Ezzedine *et al* (1991) *New Biol* 3, 608). The cytosine deaminase of any organism, for example *E. coli* or *Saccharomyces cerevisiae*, may be used.

Thus, in a preferred embodiment of the invention, the DNA construct encodes a cytosine deaminase and the patient is concomitantly given 5FC. By "concomitantly", we mean that the 5FC is administered at such a time, in relation to the transformation of the tumour cells, that 5FC is converted into 5FU in the tumour cells by the cytosine deaminase expressed from the said construct. A dosage of approximately 0.001 to 100.0 mg 5FC/kg body weight/day, preferably 0.1 to 10.0 mg/kg/day is suitable.

Other examples of pro-drug/enzyme combinations include those disclosed by Bagshawe *et al* (WO 88/07378), namely various alkylating agents and the *Pseudomonas* spp. CPG2 enzyme, and those disclosed by Epenetos & Rowlinson-Busza (WO 91/11201), namely cyanogenic pro-drugs (for example amygdalin) and plant-derived β-glucosidases.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumour cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form (see, for example, D.E.V. Wilman "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions* 14, 375-382 (615th Meeting, Belfast 1986) and V.J. Stella *et al* "Prodrugs: A Chemical Approach to Targeted Drug Delivery" *Directed Drug Delivery* R. Borchardt *et al* (ed.) pages 247-267 (Humana Press 1985)).

The enzymatic activation of prodrugs results in a large number of drug molecules generated per conjugate molecule which can then diffuse to tumour regions previously inaccessible to the conjugate. Furthermore, not all of the tumour cells in a given population need to bind the enzyme conjugate in order to be affected by the cytotoxic agent.

Several factors need to be taken into account in selecting an enzyme for prodrug activation. These include the molecular weight and physical properties of the enzyme, its activity and stability under physiological conditions, and the nature of the drug that the enzyme generates.

The adaptability of the strategy caters for the employment of a variety of enzymes which have the potential to release a multitude of mechanistically separate anticancer agents. Of particular value is the fact that a single Mab-enzyme conjugate can generate therapeutically effective doses of mechanistically

distinct anticancer agents possessing synergistic activities. This should prove important for immunogenicity reasons. In these respects, many β-lactamases hold a great deal of potential because of their favourable kinetics and broad substrate specificities, as well as their abilities to effect the elimination of substituents appended to the 3'-position of cephalosporin substrates (see Svensson *et al* (1992) "Mab-β-lactamase conjugates for the activation of a cephalosporin mustard prodrug" *Bioconjugate Chem.* 3, 176-181).

Enzymes of both mammalian and non-mammalian origin are currently being explored for the activation of a wide range of prodrugs (Senter *et al*, 1993. Generation of cytotoxic agents by targeted enzymes. Bioconjugate 4, 3-9; Senter *et al*, 1991. Activation of prodrugs by antibody-enzyme conjugates. *In* Immunobiology of Proteins and Peptides VI, ed. M.Z.Atassi. Plenum Press, New York, pp 97-105). While enzymes of mammalian origin might be advantageous due to reduced immunogenicity, the prodrugs that they act upon might be substrates for corresponding endogenous enzymes.

Enzymes that are useful in the method of this invention include, but are not limited to alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs, arylsulphatase useful for converting sulphate-containing prodrugs into free drugs, cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil, proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs, D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents, carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs, β-lactamase useful for converting drugs derivatized with β-lactams into free drugs, and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for

converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as abzymes, can be used to convert the prodrugs of the invention into free active drugs [see, e.g. R J Massey, *Nature*, 328, pp. 457-458 (1987)]. Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumour cell population.

Similarly, the prodrugs of this invention include, but are not limited to, the above-listed prodrugs, e.g., phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulphate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted by the enzyme of the conjugate into the more active, cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, etoposide, teniposide, adriamycin, daunomycin, carminomycin, aminopterin, dactinomycin, mitomycins, cis-platinum and cis-platinum analogues, bleomycins, esperamicins [see U.S. Pat. No. 4,675,187], 5-fluorouracil, melphalan and other related nitrogen mustards.

The constructs of the invention may be introduced into the tumour cells by any convenient method, for example methods involving retroviruses, so that the construct is inserted into the genome of the tumour cell. For example, in Kuriyama *et al* (1991) *Cell Struc. and Func.* **16**, 503-510 purified retroviruses are administered. Retroviruses provide a potential means of selectively infecting cancer cells because they can only integrate into the genome of dividing cells; most normal cells surrounding cancers are in a quiescent, non-receptive stage of cell growth. Retroviral DNA constructs which contain a

promoter segment which specifically binds the c-*erb*B-2-binding nuclear transcription activator and a heterologous coding sequence may be made using methods well known in the art. To produce active retrovirus from such a construct it is usual to use an ecotropic psi2 packaging cell line grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS). Transfection of the cell line is conveniently by calcium phosphate co-precipitation, and stable transformants are selected by addition of G418 to a final concentration of 1 mg/ml (assuming the retroviral construct contains a neo^R gene). Independent colonies are isolated and expanded and the culture supernatant removed, filtered through a 0.45 μm pore-size filter and stored at -70°. For the introduction of the retrovirus into the tumour cells, it is convenient to inject directly retroviral supernatant to which 10 μg/ml Polybrene has been added. For tumours exceeding 10 mm in diameter it is appropriate to inject between 0.1 ml and 1 ml of retroviral supernatant; preferably 0.5 ml. Alternatively, as described in Culver *et al* (1992) *Science* 256, 1550-1552, cells which produce retroviruses are injected into the tumour. The retrovirus-producing cells so introduced are engineered to actively produce retroviral vector particles so that continuous productions of the vector occurred within the tumour mass *in situ*. Thus, proliferating tumour cells can be successfully transduced *in vivo* if mixed with retroviral vector-producing cells. Other methods involve simple delivery of the construct into the cell for expression therein either for a limited time or, following integration into the genome, for a longer time. An example of the latter approach includes (preferably tumour-cell-targeted) liposomes (Nässander *et al* (1992) *Cancer Res.* 52, 646-653).

Immunoliposomes (antibody-directed liposomes) are especially useful in targeting to cancer cell types which over-express a cell surface protein for which antibodies are available (see Table for examples). In relation to the present invention, antibodies directed towards the c-*erb*B-2 protein are

preferred. For the preparati n of immuno-liposomes MPB-PE (N-[4-(p-maleimidophenyl)butyryl]-phosphatidylethanolamine) is synthesised according to the method of Martin & Papahadjopoulos (1982) *J. Biol. Chem.* 257, 286-288. MPB-PE is incorporated into the liposomal bilayers to allow a covalent coupling of the antibody, or fragment thereof, to the liposomal surface. The liposome is conveniently loaded with the DNA construct of the invention for delivery to the target cells, for example, by forming the said liposomes in a solution of the DNA construct, followed by sequential extrusion through polycarbonate membrane filters with 0.6 μm and 0.2 μm pore size under nitrogen pressures up to 0.8 MPa. After extrusion, entrapped DNA construct is separated from free DNA construct by ultracentrifugation at 80 000 x g for 45 min. Freshly prepared MPB-PE-liposomes in deoxygenated buffer are mixed with freshly prepared antibody (or fragment thereof) and the coupling reactions are carried out in a nitrogen atmosphere at 4°C under constant end over end rotation overnight. The immunoliposomes are separated from unconjugated antibodies by ultracentrifugation at 80 000 x g for 45 min. Immunoliposomes may be injected intraperitoneally or directly into the tumour.

Table

1. Tumour Associated Antigens

Antigen	Antibody	Existing uses
Placental Alkaline Phosphatase	H17E2 (ICRF, Travers & Bodmer)	Imaging and therapy of testicular and ovarian cancers.
Pan Carcinoma	NR-LU-10 (NeoRx Corporation)	Imaging and therapy of various carcin-omas including small cell lung cancer.

Polymorphic Epithelial Mucin (Human milk fat globule)	HMFG1 (Taylor-Papadimitriou, ICRF)	Imaging and therapy of ovarian cancer and pleural effusions.
A carbohydrate on Human Carcinomas	L6 (IgG2a)[1]	Targeting of alkaline phosphatase (Senter *et al* (1988) *PNAS USA* 85, 4842-4846.

[1] Hellström *et al* (1986) *Cancer Res.* 46, 3917-3923

Antibodies to the polymorphic epithelial mucin antigen are preferred.

It will be appreciated that monoclonal antibodies or other molecules that bind to tumour cell surface antigens such as the c-*erb*B-2 receptor, are useful in targeting the DNA construct of the invention.

Monoclonal antibodies which will bind to many of these antigens are already known but in any case, with today's techniques in relation to monoclonal antibody technology, antibodies can be prepared to most antigens. The antigen-binding portion may be a part of an antibody (for example a Fab fragment) or a synthetic antibody fragment (for example a single chain Fv fragment [ScFv]). Suitable monoclonal antibodies to selected antigens may be prepared by known techniques, for example those disclosed in *"Monoclonal Antibodies: A manual of techniques"*, H Zola (CRC Press, 1988) and in *"Monoclonal Hybridoma Antibodies: Techniques and Applications"*, J G R Hurrell (CRC Press, 1982).

Chimaeric antibodies are discussed by Neuberger *et al* (1988, *8th International Biotechnology Symposium* Part 2, 792-799).

Suitably prepared non-human antibodies can be "humanized" in known ways, for example by inserting the CDR regions of mouse antibodies into the

framework of human antibodies. Such "humanized" antibodies, or fragments thereof, are preferred as they may give rise to a lower anti-antibody reaction than rodent antibodies.

The variable heavy (V_H) and variable light (V_L) domains of the antibody are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation" of rodent antibodies. Variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parented antibody (Morrison *et al* (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6851-6855).

That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better *et al* (1988) *Science* **240**, 1041); Fv molecules (Skerra *et al* (1988) *Science* **240**, 1038); single-chain Fv (ScFv) molecules where the V_H and V_L partner domains are linked via a flexible oligopeptide (Bird *et al* (1988) *Science* **242**, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward *et al* (1989) *Nature* **341**, 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) *Nature* **349**, 293-299.

By "ScFv molecules" we mean molecules wherein the V_H and V_L partner domains are linked via a flexible oligopeptide.

The advantages of using antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved

pharmacological properties, such as better penetration of solid tissue. Effector functions of whole antibodies, such as complement binding, are removed. Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of the said fragments.

Whole antibodies, and $F(ab')_2$ fragments are "bivalent". By "bivalent" we mean that the said antibodies and $F(ab')_2$ fragments have two antigen combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining sites.

Other molecules immunologically reactive with the target cell surface molecule are also useful in this aspect of the invention and include, for example minimal recognition units (MRU) and complementarity determining regions.

Other methods of delivery include adenoviruses carrying external DNA via an antibody-polylysine bridge (see Curiel *Prog. Med. Virol.* 40, 1-18) and transferrin-polycation conjugates as carriers (Wagner *et al* (1990) *Proc. Natl. Acad. Sci. USA* 87, 3410-3414). In the first of these methods a polycation-antibody complex is formed with the DNA construct of the invention, wherein the antibody is specific for either wild-type adenovirus or a variant adenovirus in which a new epitope has been introduced which binds the antibody. The polycation moiety binds the DNA via electrostatic interactions with the phosphate backbone. The adenovirus, because it contains unaltered fibre and pentos proteins, is internalized into the cell and carries into the cell with it the DNA construct of the invention. It is preferred if the polycation is polylysine.

In the second of these methods, a high-efficiency nucleic acid delivery system that uses receptor-mediated endocytosis to carry DNA macromolecules into cells is employed. This is accomplished by conjugating the iron-transport

protein transferrin to polycations that bind nucleic acids. Human transferrin, or the chicken homologue conalbumin, or combinations thereof is covalently linked to the small DNA-binding protein protamine or to polylysines of various sizes through a disulfide linkage. These modified transferrin molecules maintain their ability to bind their cognate receptor and to mediate efficient iron transport into the cell. The transferrin-polycation molecules form electrophoretically stable complexes with DNA constructs of the invention independent of nucleic acid size (from short oligonucleotides to DNA of 21 kilobase pairs). When complexes of transferrin-polycation and the DNA constructs of the invention are supplied to the tumour cells, a high level of expression from the construct in the cells is expected.

High-efficiency receptor-mediated delivery of the DNA constructs of the invention using the endosome-disruption activity of defective or chemically inactivated adenovirus particles produced by the methods of Cotten *et al* (1992) *Proc. Natl. Acad. Sci. USA* 89, 6094-6098 may also be used. This approach appears to rely on the fact that adenoviruses are adapted to allow release of their DNA from an endosome without passage through the lysosome, and in the presence of, for example transferrin linked to the DNA construct of the invention, the DNA construct is taken up by the cell by the same route as the adenovirus particle.

It may be desirable to locally perfuse a tumour with the delivery vehicle (for example the retrovirus) for a period of time.

Ligands for the c-*erb*B-2 receptor kinase have been identified and the nucleotide sequence of cDNAs and amino acid sequences derived therefrom have been published by Holmes *et al* (1992) *Science* 256, 1205-1210 incorporated herein by reference. Thus, it is preferred that the ligand (known as heregulin) is used to target the DNA constructs of the invention to the cells that over-express c-

*erb*B-2.

In one embodiment an active, truncated c-*erb*B-2 ligand is synthesised and is coupled to a poly-lysine chain. These molecules are then mixed with the DNA construct of the invention, allowing the lysine chains to bind the DNA. These protein-DNA complexes then bind specifically to cells expressing c-*erb*B-2 through interaction with the ligand. The ensuing receptor internalisation causes the complexed DNA to enter the cell and expression of the encoded enzyme would commence (Figure 9).

This approach has the advantages that there is no need to use complex retroviral constructs with their attendant expression problems; there is no permanent modification of the genome as occurs with retroviral infection; and the targeted expression system is coupled with a targeted delivery system, thus reducing toxicity to other cell types.

The whole heregulin molecule may be used for targeting, but it is preferred if a 65 amino acid portion of heregulin, which is fully active in binding to c-*erb*B-2 is used. Of course, fragments and variants of heregulin that retain the ability to bind c-*erb*B-2 may be used for targeting.

It is further preferred if the heregulin, or fragment or variant thereof, is incorporated into liposomes so as to be displayed on the liposome surface and that the liposome further contains the DNA construct of the invention.

The 65 amino acid portion extends from serine 177 to tyrosine 241 of heregulin HRG-β1, but other homologous portions of the HRG-α, HRG-β2 and HRG-β3 heregulins may also be used. These amino acid sequences are described in Holmes *et al* (1992) *Science* **256**, 1205-1210 incorporated herein by reference.

Conveniently, the portion of heregulin useful in targeting c-*erb*B-2 is produced by expressing a cognate DNA sequence. Thus, a gene is synthesised which encodes the desired portion of heregulin; the sequence of the gene is readily determined using the known amino acid sequences and common knowledge of the genetic code. Because of the degeneracy of the genetic code, it is preferred to choose a codon for each amino acid which is most favoured by the organism in which the DNA is to be expressed.

The DNA is then expressed in a suitable host to produce a polypeptide ligand. Thus, the DNA encoding the polypeptide ligand may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the polypeptide of the invention. Such techniques include those disclosed in US Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crowl, 4,677,063 issued 30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

The DNA encoding the polypeptide ligand may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary,

the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can then be recovered.

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

The vectors include a procaryotic replicon, such as the ColE1 ori, for propagation in a procaryote, even if the vector is to be used for expression in other, non-procaryotic, cell types. The vectors can also include an appropriate promoter such as a procaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E. coli*, transformed therewith.

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter

sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

Typical procaryotic vector plasmids are pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and pTrc99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.

A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells.

An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers his3, trp1, leu2 and ura3. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps)

A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3′-single-stranded termini with their 3′-5′-exonucleolytic activities, and fill in recessed 3'-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the DNA encoding the polypeptide of the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491.

In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

The host cell can be eith r procaryotic or eucaryotic. Bacterial cells are preferred procaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eucaryotic host cells include yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, and monkey kidney-derived COS-1 cells available from the ATCC as CRL 1650; preferred insect cells are Lepidoptera insect cells such as Sf9 cells for use with baculovirus expression systems. The use of insect cells to express gene products is reviewed in Fraser (1992) *Curr. Top. Microbiol. Immunol.* **158**, 131-172.

Transformation of appropriate cell hosts with a DNA construct is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of procaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2110 and Sambrook *et al* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* **275**, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

Successfully transformed cells, ie cells that contain a DNA construct, can be identified by well known techniques. For example, cells resulting from the introduction of the expression construct of can be grown to produce the polypeptide ligand. Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.

Alternatively, peptide ligands may be synthesised by the Fmoc-polyamide mode of solid-phase peptide synthesis as disclosed by Lu *et al* (1981) *J. Org. Chem.* **46**, 3433 and references therein. Temporary N-amino group protection is afforded by the 9-fluorenylmethyloxycarbonyl (Fmoc) group. Repetitive cleavage of this highly base-labile protecting group is effected using 20% piperidine in N,N-dimethylformamide. Side-chain functionalities may be protected as their butyl ethers (in the case of serine threonine and tyrosine), butyl esters (in the case of glutamic acid and aspartic acid), butyloxycarbonyl derivative (in the case of lysine and histidine), trityl derivative (in the case of cysteine) and 4-methoxy-2,3,6-trimethylbenzenesulphonyl derivative (in the case of arginine). Where glutamine or asparagine are C-terminal residues, use is made of the 4,4'-dimethoxybenzhydryl group for protection of the side chain amido functionalities. The solid-phase support is based on a polydimethyl-acrylamide polymer constituted from the three monomers dimethylacrylamide (backbone-monomer), bisacryloylethylene diamine (cross linker) and acryloylsarcosine methyl ester (functionalising agent). The peptide-to-resin cleavable linked agent used is the acid-labile 4-hydroxymethyl-phenoxyacetic acid derivative. All amino acid derivatives are added as their preformed symmetrical anhydride derivatives with the exception of asparagine and glutamine, which are added using a reversed N,N-dicyclohexyl-carbodiimide/1-hydroxybenzotriazole mediated coupling procedure. All coupling and deprotection reactions are monitored using ninhydrin, trinitrobenzene sulphonic acid or isotin test procedures. Upon completion of synthesis, peptides are cleaved from the resin support with concomitant removal of side-chain

pr tecting groups by treatment with 95% trifluoroacetic acid containing a 50% scavenger mix. Scavengers commonly used are ethanedithiol, phenol, anisole and water, the exact choice depending on the constituent amino acids of the peptide being synthesised. Trifluoroacetic acid is removed by evaporation in vacuo, with subsequent trituration with diethyl ether affording the crude peptide. Any scavengers present are removed by a simple extraction procedure which on lyophilisation of the aqueous phase affords the crude peptide free of scavengers. Reagents for peptide synthesis are generally available from Calbiochem-Novabiochem (UK) Ltd, Nottingham NG7 2QJ, UK. Purification may be effected by any one, or a combination of, techniques such as size exclusion chromatography, ion-exchange chromatography and (principally) reverse-phase high performance liquid chromatography. Analysis of peptides may be carried out using thin layer chromatography, reverse-phase high performance liquid chromatography, amino-acid analysis after acid hydrolysis and by fast atom bombardment (FAB) mass spectrometric analysis.

The coupling of poly-lysine to peptides via a bifunctional reagent has been described by Wagner *et al* (1990) above in the context of joining poly-lysine to transferrin. This reaction uses lysine residues within the protein sequence which might not be desirable here as a loss of function could result. However, this is overcome by engineering additional lysine residues at the C terminus of the heregulin fragments as they would be more exposed than lysine residues in the body of the protein.

The specificity of using such heregulin fragments for targeting the DNA constructs of the invention to c-*erb*B-2 over-expressing cells is demonstrated by the finding that heregulin has no ability to bind the highly homologous EGF receptor protein (Holmes *et al* (1992) *Science* **256**, 1205-1210).

In a further preferred embodiment, heregulin or a c-*erb*B-2 receptor-binding

fragment thereof, linked to the DNA construct of the invention via poly-lysine, is mixed with inactivated adenovirus particles and used in the ways described by Cotten *et al* (1992) *Proc. Natl. Acad. Sci. USA* 89, 6094-6098.

Any tumour in which the promoter is over-expressed and to which the construct of the invention can be administered may be attacked. We have found that the promoter directs over-expression in breast, ovarian, pancreatic and gastric cancers. Thus, these tumour types may be diagnosed and treated using the methods and compounds of the invention. It is preferred if the tumour type is a breast tumour or pancreatic tumour.

The promoter, when in at least some breast cancer cell lines, has been found to direct overexpression very effectively, and the nuclear transcription factor is abundant in at least some breast cancer cell lines. Thus, it is preferred that the construct of the invention is administered to breast cancers.

It is known that tumours, including breast tumours, can metastasise and that cells of the primary tumour may develop into secondary tumours at sites remote from the primary tumour site. The construct of the invention may be useful in treating such secondary tumours, particularly if such secondary tumours are derived from primary breast cancer cells.

One aspect of the invention provides a purified nuclear transcription factor that binds to the SEQ ID No. 1 sequence of the c-*erb*B-2 promoter.

As described in more detail in Example 2 below, we have identified a nuclear transcription factor that binds to the c-*erb*B-2 promoter. The factor has been shown by Western blotting to be more abundant in nuclear extracts derived from c-*erb*B-2 over-expressing cell lines using antibodies raised to the factor (see Example 11). We have called this factor OB2-1 and have sh wn that it

binds to the sequence SEQ ID No. 1. Purification and peptide sequences of OB2-1 and antibodies directed towards OB2-1 are described in detail in the examples.

Fragments or variants of OB2-1 are also useful in this aspect of the invention, especially those that are immunologically cross-reactive therewith. Thus, one embodiment of the invention provides variants of OB2-1 in which amino acid residues have been conservatively substituted.

By "conservatively substituted" we mean the accepted point mutations in 71 families of related proteins as disclosed by Schwartz & Dayhoff (1978) in *Atlas of Protein Sequence and Structure* (Dayhoff, M.D. ed), Vol. 5, Supplement 3, pp 353-358, National Biomedical Research Foundation, Washington.

Identification and quantitation of the nuclear transcription factor of the invention has several uses, particularly in the field of clinical management of cancer patients, especially breast cancer patients. It is well known that the c-*erb*B-2 receptor tyrosine kinase proto-oncogene product is over-expressed in 20-30% of breast carcinomas and this has been shown to correlate with poor prognosis. In this work we show that c-*erb*B-2-over-expressing cell lines contain more nuclear transcription factor than do non-over-expressing cell lines. Thus, a comparison of the abundance of nuclear transcription factor in over-expressing and non-expressing cells derived from the tumour provides a means to aid diagnosis and prognosis of cancer cells, and for determining whether the tumour is suitable for treatment herein described. The clinician will find the results of such a test useful in helping in diagnosis and prognosis but may use other tests in conjunction with the present test before reaching a conclusion.

It is preferred if the abundance of the nuclear transcription factor OB2-1 is measured.

It is further preferred if the abundance of the nuclear transcription factor is measured using antibodies which cross-react with the said transcription factor. Thus, a further aspect of the invention provides antibodies reactive against the nuclear transcription factor. Antibodies can be used to determine the abundance of the nuclear transcription factor by *in situ* staining methods or by using enzyme-linked immunosorbent assays (ELISAs).

By "antibodies" we include whole antibodies, antibody fragments such as Fab, Fv and $F(ab')_2$ fragments, and genetically engineered molecules which retain the antigen recognition site such as single chain Fv fragments (scFv) and single domain antibodies (dAb). Other molecules immunologically reactive with OB2-1 are also useful in this aspect of the invention and include, for example minimal recognition units (MRU) and complementarity determining regions (CDR).

Antibodies may readily be made by methods well known in the art and as described *supra* in relation to immunoliposomes.

A further aspect provides peptides derived from the OB2-1 transcription factor. Such peptides include:

FR43: DFAYVCEAEFPSK (SEQ ID No. 6); FR51: AAHVTLLTSLVEGEAVHLAR (SEQ ID No. 7); FR31: IGLNLPAGR (SEQ ID No. 8); FR38: ELVGAVMNPTEVFX (SEQ ID No. 9); FR23: QSQESGLLXTHR (SEQ ID No. 10); FRXX: LSLLSSTSK (SEQ ID No. 11); FR45: DNLFGGVVNPNEVFC (SEQ ID No. 12); FR46: LSPPECLNASLLGGVLR (SEQ ID No. 13); FR47 LSPP (SEQ ID No. 14); FR63: GLPHQQSGL (SEQ ID No. 15); FR39a: ELVEAVMNPS (SEQ ID No. 30); FR39b: ELVGAVMNPS (SEQ ID No. 31); XFLNNTTTNH (SEQ ID No. 32); FR46 top: LSPPECHNASLLGGVLR (SEQ ID No. 33); and

XXXLLSHAQXPYSDAPG (SEQ ID N . 34).

The peptides of the invention are useful in raising antibodies directed towards OB2-1. Peptides in which one or more of the amino acid residues are chemically modified, before or after the peptide is synthesised, may be used providing that the function of the peptide, namely the production of specific antibodies *in vivo*, remains substantially unchanged. Such modifications include forming salts with acids or bases, especially physiologically acceptable organic or inorganic acids and bases, forming an ester or amide of a terminal carboxyl group, and attaching amino acid protecting groups such as N-t-butoxycarbonyl. Such modifications may protect the peptide from *in vivo* metabolism. The peptides may be present as single copies or as multiples, for example tandem repeats. Such tandem or multiple repeats may be sufficiently antigenic themselves to obviate the use of a carrier. It may be advantageous for the peptide to be formed as a loop, with the N-terminal and C-terminal ends joined together, or to add one or more Cys residues to an end to increase antigenicity and/or to allow disulphide bonds to be formed. If the peptide is covalently linked to a carrier, preferably a polypeptide, then the arrangement is preferably such that the peptide of the invention forms a loop.

According to current immunological theories, a carrier function should be present in any immunogenic formulation in order to stimulate, or enhance stimulation of, the immune system. It is thought that the best carriers embody (or, together with the antigen, create) a T-cell epitope. The peptides may be associated, for example by cross-linking, with a separate carrier, such as serum albumins, myoglobins, bacterial toxoids and keyhole limpet haemocyanin. More recently developed carriers which induce T-cell help in the immune response include the hepatitis-B core antigen (also called the nucleocapsid protein), presumed T-cell epitopes such as Thr-Ala-Ser-Gly-Val-Ala-Glu-Thr-Thr-Asn-Cys (SEQ ID No. 16), beta-galactosidase and the 163-171 peptide of

interleukin-1. The latter compound may variously be regarded as a carrier or as an adjuvant or as both. Alternatively, several copies of the same or different peptides of the invention may be cross-linked to one another; in this situation there is no separate carrier as such, but a carrier function may be provided by such cross-linking. Suitable cross-linking agents include those listed as such in the Sigma and Pierce catalogues, for example glutaraldehyde, carbodiimide and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, the latter agent exploiting the -SH group on the C-terminal cysteine residue (if present). However, as is described in more detail below, peptides which are found in AP-2 but not AP-2B are most preferred.

A further aspect provides a DNA molecule encoding the OB2-1 transcription factor, or fragment or variant thereof.

A DNA molecule encoding the OB2-1 transcription factor is isolated using standard molecular biology techniques as described in Sambrook *et al* (1989) *Molecular Cloning: A laboratory manual*, Cold Spring Harbor Press, Cold Spring Harbor, New York. Typically, a probe, specific for the cDNA encoding OB2-1, is radiolabelled with ^{32}P and used to screen a cDNA library.

The cDNA library is derived from mRNA isolated from a cell line abundant in OB2-1 such as ZR 75-1, and the probe is a degenerate oligonucleotide, the sequence of which is readily determined from the peptides of the invention using the genetic code. Alternatively, the probe is generated by the polymerase chain reaction using a pair of oligonucleotide primers whose sequences are determined by the peptides of the invention, such that one degenerate primer hybridises to the sense strand of OB2-1 cDNA, and the other primer hybridises to the antisense strand. Alternatively, as described below, AP2 cDNA may be used as a probe to isolate OB2-1 cDNAs.

The nucleotide sequence f the cDNA is determined using the dideoxy chain termination method, and the amino acid sequence is deduced.

It will be appreciated that the level of OB2-1 may be determined in tumour cells by measuring the abundance of the cognate mRNA rather than the protein. Accordingly, in one embodiment the level of mRNA is quantitated using a nucleic acid probe directed to the mRNA, or by using a reverse transcriptase-polymerase chain reaction (RT-PCR). However, it is preferred if the abundance of the polypeptide is measured.

A further aspect of the invention provides a method of predicting which tumours are particularly suited to treatment using the DNA construct of the invention. Samples of tumour tissue are taken and the abundance of OB2-1 is measured using the antibody or nucleic acid methods described above. The abundance of OB2-1 protein or mRNA is compared to the level of a marker protein or marker mRNA that remains substantially constant in most cell types, for example actin. If, in comparison with non-tumour cells (derived from the same tissue type as the tumour cells) the abundance of OB2-1 is increased in the tumour cells, then these tumour cells are particularly suited to treatment with the DNA constructs of the invention.

By "abundance of OB2-1 is increased" we mean that in tumour cells, compared with the non-tumour cells, the level of OB2-1 compared to the substantially constant marker is between two-fold and twenty-fold higher; preferably between three-fold and ten-fold higher.

We have found, by comparing the peptide sequences derived from OB2-1 with other known proteins, that OB2-1 is closely related in sequence to the transcription factor AP2, and may, in fact, be equivalent to AP-2.

For example, peptide FR43 from OB2-1 differs in only three positions from the peptide DFGYVCETEFPAK (SEQ ID No. 17) derived from AP2 (the cDNA sequence of AP2 is disclosed in Williams *et al* (1988) *Genes & Develop.* 2, 1557-1569). AP2 is a sequence-specific DNA-binding protein, that does not appear to be a zinc-finger protein, that interacts with inducible viral and cellular enhancer elements to stimulate transcription of selected genes; it has previously been implicated in the control of developmentally-regulated gene expression (Luscher *et al* (1989) *Genes & Develop.* 3, 1507-1517; Mitchell *et al* (1991) *Gene & Develop.* 5, 105-119) and in epidermal-specific gene expression (Leask *et al* (1991) *Proc. Natl. Acad. Sci. USA* 88, 7948-7952) but there has been no suggestion that it plays a role in breast, ovarian, pancreatic or gastric cancers, or that its abundance is increased in cancerous cells derived from these tissues compared to non-cancerous cells.

Thus, AP2 has not been sought nor detected previously in tumour tissue, including breast tumour tissue, but antibodies which react with AP2 are useful in the screening methods described above as they may also react with OB2-1. Antibodies which show greater specificity to OB2-1 than AP2 may be obtained using the peptide FR43, which shows three amino acid changes to the corresponding AP2 peptide, as an immunogen. However, this difference in sequence may not be of significance.

An antibody towards AP2 which may be useful in determining the abundance of OB2-1 if it reacts thereto is described by Lüscher *et al* (1989) *Genes & Develop.* 3, 1507-1517 and can be made as follows.

The amino-terminal peptide KLTDNIKYEDCEDRHDGTS (SEQ ID No. 18), corresponding to AP2 amino acids 4-22, is synthesised and coupled to TPPD before injection into rabbits. Antibodies directed against AP2 are partially purified from the rabbit polyclonal antisera by passage over an Affigel-10

c lumn (Bio-Rad) to which the AP2 peptide had been coupled according to the manufacturer's recommendations. Bound antibody is eluted using 0.1M glycine-HCl (pH 2.3) and immediately neutralised, using one-tenth volume of 2M Tris-HCl (pH 7.9).

AP-2 is apparently encoded by a single gene in humans and produces transcripts in the 3.5 kd range. The original (full length) AP-2 cDNA was published by Williams *et al* (1988) *Genes & Develop.* 2, 1557-1569. The AP-2B cDNA was published by Buettner *et al* (1993) *Mol. Cell. Biol.* 13, 4174-4185 and is produced from a differently spliced message from the same gene.

As shown diagrammatically in Figure 19, the first 295 amino acids (ie the N-termini) of the two proteins are identical, but their C-termini are encoded by different exons and are completely distinct. The two sequences diverge just after the DNA binding domain such that AP-2B lacks the dimerisation domain of AP-2. Consequently, AP-2B cannot bind DNA as these proteins need to be dimers to bind. The role - if any - of AP-2B is unclear.

By Western Blotting analysis, we can find AP-2B protein in all mammary cell lines, but the full length active protein is only found in lines which overexpress c-*erb*B-2. We have also used functional assays to show that AP-2 can specifically activate the c-*erb*B-2 promoter. Therefore, AP-2 alone correlates with activation of c-*erb*B-2.

Antibodies raised to the unique C-terminal sequences of AP-2 distinguish the two proteins.

Thus, it is preferred if the antibodies used in the methods of the invention are directed towards peptides found in the region from amino acid 295 to amino acid 437 in full length AP-2 protein.

Affinity purified rabbit antiserum to the C-terminal peptide NH_2-SHTDNNAKSSDKEEKHRK-COOH (SEQ ID No 35) are commercially available from Santa Cruz Biotechnology, Santa Cruz, California, USA.

Of course, other peptides from this region of the AP-2 protein can be used to raise antibodies, both monoclonal and polyclonal, using methods well known in the art.

Preferred peptides for raising such antibodies are: NH_2-RQHSDPNEQVIRKN-COOH SEQ ID No 36 and NH_2-LAQDRSPLGNSRP-COOH SEQ ID No 37.

The similarity between OB2-1 and AP21 at the peptide sequence level suggests that the cDNA which encodes OB2-1 may be sufficiently similar to AP2 to be able to use the AP2 cDNA as a nucleic acid probe to isolate OB2-1 cDNA.

We have found that the overexpression of c-*erb*B-2 in breast cancer cells is associated with an increased abundance of the nuclear transcription activator OB2-1 in those cells, and that binding of OB2-1 to the c-*erb*B-2 promoter is an important event in the overexpression of c-*erb*B-2. We have unexpectedly found that interference of the binding of OB2-1 to the c-*erb*B-2 promoter by gold-containing compounds leads to a reduction in the expression of c-*erb*B-2 (see Example 12).

Thus, a further aspect of the invention provides a method of treating cancer in a patient comprising administering to a patient a tumour-inhibiting amount of a compound that substantially prevents the binding of OB2-1 to its DNA binding site.

Gold-containing compounds such as aurothiomalate, aurothioglucose and auranofin are well known in the treatment of rheumatoid arthritis; and

auranofin and other gold phosphino compounds have been tested as anti-tumour agents, including against mammary adenocarcinoma (US 4857549). Aurothiomalate has been shown to interfere with the binding of progesterone receptor (a zinc-finger protein), in breast cell nuclear extracts, with its DNA response element (Handel *et al* (1991) *Mol. Pharmacol.* **40**, 613-618), but there has been no suggestion that aurothiomalate would be useful in the treatment of cancer, including breast cancer.

Thus, a preferred embodiment provides a method of treating cancer in a patient comprising administering to the patient a tumour-inhibiting amount of a gold compound of the general formula

Au-X-R

where X is a linking group and R is a water soluble moiety. Preferably X is S, and R is a sugar moiety or an organic acid.

In further preference gold is in the Au(I) oxidation state.

Preferred compounds include aurothiomalic acid or a pharmaceutically acceptable salt thereof, and aurothioglucose.

Other preferred compounds include pharmaceutically acceptable compositions of Zn^{2+} or Cd^{2+}, and aromatic C-nitroso compounds such as 3-nitrosobenzamide and 6-nitroso-1,2-benzopyrone.

It is further preferred if it is determined whether the cancer to be treated is a cancer in which the abundance of OB2-1 is increased.

It is preferred if the cancer to be treated is breast cancer.

Tumours which have an increased abundance of OB2-1 may be particularly

susceptible to the gold compounds of the invention and also to other gold compounds which have previously been used to treat cancer, such as auranofin and other gold phosphino compounds.

Thus, a further aspect of the invention provides a method of predicting which tumours are particularly suited to treatment with gold compounds. Samples of tumour tissue are taken and the abundance of OB2-1 is measured using the antibody or nucleic acid methods described above. The abundance of OB2-1 protein or mRNA is compared to the level of a marker protein or marker mRNA that remains substantially constant in most cell types, for example actin. If, in comparison with non-tumour cells (derived from the same tissue type as the tumour cells) the abundance of OB2-1 is increased in the tumour cells, then these tumour cells are particularly suited to treatment with the DNA constructs of the invention.

The gold compounds are administered by any convenient route, but most usually by intratumoural injection. Pharmaceutical compositions of the compounds are typically made in sterile, pyrogen free water. In the case of compounds which are acids, it is convenient to supply them as pharmaceutically-acceptable salts of alkali metals, alkaline earth metals or amines, particularly quaternary alkylamines. In the case of aurothiomalate the sodium salt is preferred.

Typically doses of 10 mg or 20 mg are given to test the patient's tolerance to the compounds; thereafter doses of 50 mg are given, preferably at weekly intervals, although the dose and frequency of administration may be varied depending on the outcome of the treatment.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such

methods include the step of bringing into association the active ingredient (compound of the invention) with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations in accordance with the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (eg povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (eg sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethylcellulose in varying proportions to provide desired release profile.

Formulations suitable for topical administration in the mouth include lozenges

comprising the active ingredient in a flavoured basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouth-washes comprising the active ingredient in a suitable liquid carrier.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of an active ingredient.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

The c-*erb*B-2 receptor tyrosine kinase proto-oncogene is overexpressed in 25-30% of human breast carcinomas and is a marker of poor prognosis and an indicator of a poor response to conventional therapy, including endocrine therapy. Cell lines engineered to overexpress the c-*erb*B-2 protein have previously been shown to develop a tumorigenic phenotype which can be

reversed by additi n of specific antisera to the c-*erb*B-2 xtracellular domain. These antisera will also inhibit the growth of breast xenograft models. Transgenic mice which express c-*erb*B-2 in the mammary gland have also been shown to develop bilateral, highly metastatic breast carcinomas with high frequency.

In breast cancer patients, overexpression is associated with amplification of the c-*erb*B-2 gene. However it also occurs in the presence of a single copy gene. Indeed, the present work shows that, whether or not the gene is amplified, there is a 6-8 fold increase in accumulation of c-*erb*B-2 mRNA in overexpressing cells. We have now shown that this phenomenon is largely due to increased transcription of the c-*erb*B-2 gene mediated by the presence in all overexpressing lines of an additional, promoter-binding transcription factor, which we have termed OB2-1.

As the increased transcription of the c-*erb*B-2 gene is absolutely dependent on bringing OB2-1 to the promoter inhibiting the activity of this factor is a probable way of down-regulating expression of c-*erb*B-2 protein which may be of use therapeutically. We have shown that aurothiomalate which disrupts the binding activity of OB2-1 will also specifically reduce c-*erb*B-2-promoter activity in a cell-based assay system (see Example 12). Therefore, OB2-1 is an ideal target protein to put through a high-throughput screen to identify further candidate small molecules able to act as specific inhibitors of OB2-1 activity which will be useful anticancer agents.

Thus, a further aspect of the invention provides an assay to determine whether a compound reduces the binding of OB2-1 to its DNA binding site. Example 12 shows the effect of aurothiomalate (AuTM) on the binding of OB2-1 binding site using gel shift assays and by measuring the effect of AuTM on the expression from the c-*erb*B-2 promoter *in vivo*. Conveniently, in the assay,

other compounds replace AuTM in the methods of Example 12, and if the compound has substantially the same effect as AuTM then it may be useful in treating cancer.

Thus one embodiment comprises an assay for a compound which reduces the binding of OB2-1 to its DNA binding site, the assay comprising the steps (1) adding the compound to purified OB2-1; (2) further adding an OB2-1 binding site; and (3) determining whether OB2-1 is bound to the OB2-1 binding site.

A further embodiment comprises an assay for a compound which reduces the activity of OB2-1, the assay comprising the steps (1) transfecting a cell with an active promoter comprising an OB2-1 binding site which drives the expression of a reporter gene; (2) adding to the said cell or a product of the said cell the compound; and (3) determining whether the activity of the said promoter is reduced.

In a preferred embodiment, a DNA construct is made wherein OB2-1 binding sites, as described herein, are linked to a basal promoter which is then inserted upstream of a suitable reporter gene. A suitable reporter gene construct is described in Figure 20. Conveniently, the basal promoter is the glutathione transferase (GST) π gene and OB2-1 binding sites are inserted therein as described in Example 9.

The GST π promoter without OB2-1 binding sites is suitable as a control.

Of course, other basal promoters may be used so long as they do not contain an OB2-1 binding site.

Suitably, the reporter gene encodes a polypeptide that can be readily assayed. The chloramphenicol acetyl transferase (CAT) gene, as described in Examples

9 and 12, may be used as the reporter gene but it is preferred if the polypeptide encoded by the gene is an enzyme that acts on a chromogenic substrate. Examples of such enzymes are β-galactosidase which can convert *o*-nitrophenyl-β-D-glucosidase to *o*-nitrophenol, a coloured compound and alkaline phosphatase which can convert *p*-nitrophenylphosphate to *p*-nitrophenol, a yellow coloured compound.

Preferably, the alkaline phosphatase is a form that can be secreted from the cell in which the DNA construct is expressed. Details of an assay of this embodiment of the invention are described in Example 13.

A further aspect provides a compound identified in the assay as being one that substantially prevents binding of OB2-1 to its DNA binding site.

For a compound to be useful in treatment it is preferred if there is at least a two-fold reduction in binding of OB2-1 to its DNA binding site (or, where appropriate a two-fold reduction in promoter activity) as determined in the above assays. More preferably the reduction is 10-fold or 100-fold. Most preferably the reduction is 500-fold or greater.

Example 1: c-*erb*B-2 promoter and reporter enzyme

Reporter enzyme gene. The bacterial chloramphenicol acetyl transferase (CAT) gene was obtained from Promega as the "pCAT-basic" vector.

The CAT reporter system is designed to allow sensitive and rapid testing for eukaryotic transcriptional regulatory sequences. This reporter system relies on the linkage of genomic DNA fragments containing putative regulatory sequences to the chloramphenicol acetyltransferase (CAT) reporter gene. Transcriptional effects upon the CAT reporter gene are detected after

transfection into cultured cells. Since CAT is a bacterial gene, levels of CAT enzyme activity in crude cell extracts can be quickly and easily assayed with little or no background from endogenous cellular gene activity. The pCAT-Basic plasmid lacks eukaryotic promoter and enhancer sequences. This allows the researcher maximum flexibility in cloning any putative regulatory sequences into the convenient multiple cloning sites. Expression of CAT activity in cells transfected with this plasmid is dependent on insertion of a functional promoter upstream from the CAT gene. Enhancer elements can be inserted upstream from the promoter or at the *Bam*HI site downstream from the CAT gene. Sequences to be tested for transcriptional activity can be cloned into the following unique sites located immediately upstream from the CAT gene: *Xba*I, *Acc*I, *Sal*I, *Pst*I, *Sph*I and *Hin*dIII. Enhancer elements can be cloned separately into the *Bam*HI site downstream from the CAT transcriptional unit. The vector also contains the gene for ampicillin resistance.

Promoter. The human c-*erb*B-2 promoter has been cloned to -500 by two groups (Ishi *et al* (1987) *Proc Natl Acad Sci USA* 84, 4374-4378; Tal *et al* (1987) *Mol Cell Biol* 7, 2597-2601) and to -1500 by a third group (Hudson *et al* (1990a) *J Biol Chem* 265, 4389-4393). We have taken oligonucleotides to 30b regions around +40 and -500 and, using PCR against human genomic DNA, recovered a 540bp fragment representing the *erb*B-2 proximal promoter. Using oligos to -1000 and -500 we then 'PCRed' out a further 500bp representing the *erb*B-2 distal promoter. The two promoter regions were fused at the *Sma*I site at -500 and the full promoter cloned upstream of the CAT gene to generate a reporter plasmid for assaying c-*erb*B-2 promoter activity in cell lines *in vitro*. Further constructs were made by either deleting 5′ regions of the promoter using convenient restriction enzyme sites, or using PCR technology, to generate a series of promoter deletion mutants linked to CAT 3′ end always +40; 5′ ends as follows: -1000, -500, -400, -300, -213, -177, -100; (Figure 1); the positions refer to the transcription initiation site of the c-*erb*B-2 gene.

Sequences flanking the 5′ side of the c-*erb*B-2 were cloned using PCR technology from human (HBL100) genomic DNA. All PCR-generated sequences were double-strand sequenced to ensure that they conformed in every respect to published c-*erb*B-2 5′ flanking sequence. Sequences from +40 to -500 were obtained using 30b oligonucleotides to the sequences +40 to +10 and -497 to -467. Sequences from -500 to -1067 were obtained using 30b oligonucleotides to the sequences -467 to -497 and -1067 to -1037. Subsequently, a fragment representing sequences -1067 to +40 of the c-*erb*B-2 5′ flanking region was generated by ligating the two pieces of amplified DNA at the *Sma*I site at -497. This piece was subcloned into the polylinker of the promoterless reporter plasmid pCATbasic (Promega) to generate p1000CAT. Further plasmids were constructed with 5′ deletions, either by using restriction enzymes [*Sma*I at -495 (p500CAT); PstI at -213] or further PCR reactions using the +40 to +10 oligonucleotide plus a further 30b oligonucleotide whose 5′ end corresponded to the desired deletion end point (hence -400, -300 and -100).

Construction of c-*erb*B-2 plasmid. The c-*erb*B-2 promoter was incorporated in the pCAT-basic plasmid to give the plasmid shown in Figure 1 by digesting the plasmid with *Xba*I and then filling the ends with Klenow fragment to create a blunt-ended vector suitable for cloning the blunt-ended PCR products.

The CAT activity from the various promoter constructs was compared to baseline activity from the promoterless CAT parent plasmid by calcium phosphate mediated DNA transfection into a number of different breast cell lines. Immortalised normal and tumour lines which have low endogenous c-*erb*B-2 expression showed little activity of the c-*erb*B-2 promoter, ie all the reporter constructs containing c-*erb*B-2 sequences generated no more CAT activity than the promoterless control plasmid. This result makes it unlikely

that c-*erb*B-2 expression is actively repressed in these cell lines (by a tumour suppressor-like activity).

Example 2: Further identification of promoter fragments

Nuclear extracts were prepared from cell lines that show either low or high endogenous c-*erb*B-2 expression. These protein extracts were allowed to bind to radiolabelled regions of the c-*erb*B-2 promoter and subjected to partial digestion with DNase 1. This DNase footprinting technique allows the localisation of protein binding sites within a promoter. Footprinting the proximal promoter revealed 3-4 protein binding sites. Most were identical, irrespective of the origin of the nuclear extract. However, one footprint which centres at -210 was only found in extracts derived from tumour cell lines which overexpress c-*erb*B-2. This footprint therefore overlaps the region of the promoter that appears to be functionally relevant for c-*erb*B-2 overexpression. This factor will be referred to as OB2-1 (for overexpression of c-*erb*B-2, factor 1). The double-stranded sequence covered by the footprint was:

```
-226 GAGAACGGCTGCAGGCAACCCAGGCGT -200
     CTCTTGCCGACGTCCGTTGGGTCCGCA
```

These sequences (SEQ ID No. 1) were synthesised as oligonucleotides and annealed to generate a double stranded oligonucleotide representing the binding site of the factor. This was labelled and used as a probe in electromobility shift assays (EMSA) which confirmed that a protein factor could specifically bind to this sequence and that this factor was either more active or more abundant (5-10 fold) in nuclear extracts derived from c-*erb*B-2 overexpressing cell lines. Further oligonucleotides were made (SEQ ID No. 19):

```
GTGTGAGAACGGCTGAATGCAACCCAGGCGTCCC
CACACTCTTGCCGACTTACGTTGGGTCCGCAGGG
```

These incorporate a double point mutation within the centre of the sequence which is presumed to contain the core binding motif. This mutated double

stranded oligo failed to compete for binding to the wild-type (wt) sequence thus the base changes are assumed to knock out binding of the OB2-1 factor. Using PCR technology, we built this mutation into the -300 CAT reporter construct so that we then had wt and mutant forms of this reporter plasmid. The wt form gave good activity in ZR-75-1 cells.

Example 3: Expression of cytosine deaminase

Cytosine deaminase gene. The *E. coli* cytosine deaminase (CD) gene has recently been cloned (Andersson *et al* (1989) *Arch Microbiol* **152**, 115-118) and may be obtained by repeating the work described in that article or using the information in Figure 10 herein to generate the cDNA using the polymerase chain reaction.

The CD gene, modified so that the GTG initiation codon of the original gene is converted to an ATG, is incorporated in a plasmid by the method of Mullen *et al* (1992) *Proc Nat Acad Sci USA* **89**, 33-37 to give a CD2 gene. The CD2 gene is now suitable for expression in mammalian cells. The nucleotide sequence of the CD2 construct is shown in Figure 10.

Gene transfer construct. We then fused the CD2 construct to a 1 kb PCR-generated fragment of DNA from the 5′ region of the human *erb*B-2 gene which includes transcription control elements involved in overexpression of the oncogene in tumour cells as described above. This fusion construct was then cloned in the vector pBabe*neo* in reverse orientation to the retroviral LTR (Figure 4). This allows the antibiotic resistance gene *neo*, used as a dominant selectable marker, to be driven by the internal SV40 promoter while the CD2 gene will be conditionally expressed from the *erb*B-2 promoter. It may be desirable to avoid the production of antisense RNA production from the LTR by using vectors which self-inactivate in the LTR once integrated in the

genome, for example the vectors described in Hantzopoulos *et al* (1989) *PNAS* **86**, 3519-3523.

This plasmid is transfected, for example using the calcium phosphate coprecipitation method (Ikenaka *et al* (1990) DNA Cell Biol. **9**, 279-286), into the ecotropic packaging cell line omegaE or psi2 cells (Mann *et al* (1983) *Cell* **33**, 153) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS) to generate replication-defective retrovirus to infect a second packaging cell line such as *psi CRIP* (Danos & Mulligan (1988) *PNAS* **84**, 6460) to generate retrovirus capable of infecting a wide host range including human cells. This retrovirus is then introduced into patients in conjunction with the administration of 5FC.

Example 4: Injection of Retrovirus-producing cells into tumour

Initial experiments are undertaken to determine if retroviral-mediated gene transfer after reimplantation of mixtures of tumour cells with vector-producing fibroblasts can be successfully accomplished *in vivo*. Mice are inoculated subcutaneously with fibrosarcoma cells mixed with either control fibroblasts that express the neomycin resistance gene (NeoR) (3T3 cells not producing retroviral vectors) or 3T3 cells that produce the NeoR retroviral vector (PA317) (Miller & Rosman (1989) *Biotechniques* **7**, 980). To measure the efficiency of gene transfer *in vivo*, the tumours are resected after 4 weeks, reestablished in culture, and then tested in a clonogenic assay for expression of the NeoR gene by culture with the neomycin analogue G418. Growth of the tumour cells, recovered from animals injected with the tumour mixed with retroviral vector-producing fibroblasts, as G418-resistant colonies in a clonogenic assay, indicates that *in vivo* gene transfer into the proliferating tumour cells has been accomplished. Southern (DNA) blot analysis for the NeoR vector and a direct

enzyme assay for neomycin phosphotransferase are used to check that the G418-resistant tumour cells are positive.

Example 5: Treatment of Tumour with Thymidine Kinase System

Mice are injected subcutaneously with mixtures of tumour cells (murine fibrosarcoma, MCA 205) and control fibroblasts (NeoR-transduced 3T3 cells, LNL6) or with fibroblasts engineered to produce HS-tk retroviral vectors (3T3 cells transduced with the HS-tk retroviral vector PA317-G1NsCTK (Genetic Therapy, Gaithersburg, Maryland, USA). Three days after cell implantation, small growing tumours become visible, and the mice are treated twice daily with ganciclovir (GCV) (150 kg/mg body weight).

Example 6: Targeting of the construct to tumour cells

Although the constructs of the invention provide for tumour-specific expression, it may be desirable also to target the constructs to tumour cells. This may be done for example, with immunoliposomes.

Materials. The human ovarian cancer cell line NIH:OVCAR-3 is available from the American Type Culture Collection (Rockville, MD); foetal calf serum and RPMI 1640 supplemented with 25mM Hepes buffer and L-glutamine from Gibco Ltd. (Paisley, United Kingdom); the monoclonal antibody OV-TL 3 from Dr L G Poels (University of Nijmegen, the Netherlands); CF from Eastman Kodak Company (Rochester, NY) purified by the method described by Ralston *et al*, (1981); PC (Egg L-α-phosphatidylcholine type V-E) cholesterol (Chol), Hepes (4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid), DTT (dithiothreitol), bovine serum albumin, and N-ethylmaleimide from Sigma Chemical Co. (St.Louis, MO); egg phosphatidyl-glycerol from Nattermann GmbH (Cologne, Federal Republic of Germany; and Hionic Fluor,

Soluene-350, and Plasmasol from Packard Instrum nt Co., Inc (Downers Grove, Il). All other reagents are of analytical grade.

Monoclonal Antibodies, Hybridomas producing the monoclonal antibodies OV-TL 3 (mouse IgG1 type) are grown in athymic nude BALB/C mice. The monoclonal antibody OV-TL 3 is directed against human ovarian carcinoma. The antibodies were purified, fragmented, and characterised as earlier described (Nässander *et al*, 1991a). $F(ab')_2$ fragments of OV-TL 3 are incubated in 20 mM DTT at pH 5.5 for at least 90 min (Nässander *et al*, 1991a). DTT is removed by applying the incubation mixture onto a Sephadex G-25 M column (PD-10; Pharmacia). Preequilibration and elution occurred with deoxygenated acetate buffer (100mM sodium acetate-88mM NaCl, pH 6.5) under nitrogen atmosphere. Fab' fragments appearing in the void volume are used immediately for covalent attachment to preformed liposomes. In order to check the completeness of the reduction of $F(ab')_2$ to Fab' by high performance liquid chromatography (TSK-3000 SW column; LKB, Bromma, Sweden) an excess of N-ethylmaleimide is added to an aliquot of the resulting protein solution (Nässander *et al* , 1991a).

Preparation of (Immuno)liposomes. MPB-PE (*N*-[4-(p-maleimidophenyl) butyryl]-phosphatidylethanolamine) is synthesized, purified, and analyzed (Nässander, 1991b; Martin & Papahadjopolous, 1982). MPB-PE is incorporated into the liposomal bilayers to allow a covalent coupling of Fab' fragments to the liposomal surface. The composition of the liposomal bilayer is PC: egg phosphatidylglycerol: Chol: MPB-PE with a molar ratio of 38.5:4:16:1.5 A mixture of appropriate amounts of lipids and cholesteryl oleoyl ether in chloroform is evaporated to dryness in a rotary evaporator at 40°C under reduced pressure. After evacuation for at least 1 h the lipid film is hydrated in Hepes buffer (20 mM Hepes-135 mM NaCl-1 mM sucrose, pH-7.4) At this stage of preparation, the lipid concentration is 110μmol/ml TL. The resulting

liposome dispersi n is sequentially extruded through polycarbonate membranes filters with 0.6μ and 0.2 μm pore size (Uni-pore; Bio-Rad, Richmond, CA) under nitrogen pressures up to 0.8 MPa (Olson *et al*, 1979). After extrusion, the MPB-PE liposomes are separated by ultracentrifugal sedimentation at 80,000 x g during 45 min. The pellet is redispersed in acetate buffer (100 mM sodium acetate, 88mM NaCl, pH 6.5) followed by flushing with nitrogen. Freshly prepared MPB-PE-liposomes in deoxygenated buffer (concentrations during incubation ranged from 6 to 10 μmol TL/ml) are mixed with freshly prepared Fab' fragments (concentrations during incubation ranged from 40 to 400 μg/ml and retroviral vector prepared as in Example 3. The coupling reaction is carried out in a nitrogen atmosphere at 4°C under constant end over end rotation overnight. Finally, the immunoliposomes are separated from unconjugated Fab' fragments by ultracentrifugal sedimentation at 80,000 x *g* during 45 min. The pellet is resuspended and washed twice with Hepes buffer (20 mM Hepes-135 mM NaCl,pH 7.4). Liposome dispersions are stored at 4°C under nitrogen atmosphere and used within 1 week after preparation.

The retrovirus-bearing immunoliposomes are then injected into the patient intravenously.

Example 7: Preparation of cytosine deaminase cDNA from yeast

Oligonucleotide probes containing codons for a portion of the determined amino acid sequence (see EP 402 108) can be prepared and used to screen genomic or cDNA libraries for the gene encoding CDase. Basic strategies for preparing oligonucleotide probes and DNA libraries, as well as their screening by nucleic acid hybridisation, are well known to those of ordinary skill in the art. See, eg "*Oligonucleotide Synthesis*" and "*Molecular Cloning: A Laboratory Manual*". Once a clone from the screened library has been identified by positive hybridisation, restriction enzyme analysis and DNA sequencing can be

done to confirm that the particular library insert contains the gen encoding CDase. Additionally, the polymerase chain reaction (PCR) can be used to amplify and subsequently detect the nucleotide sequence coding for CDase. This method is described in Saiki *et al* (1986) and in US Patent Nos. 4 683 195 and 4 683 202, the disclosures of which are incorporated herein by reference. Analysis of the nucleotide sequence of the PCR-amplified products can be accomplished by direct sequence analysis as described by Saiki *et al* (1988). Alternatively, the amplified target sequence(s) can be cloned prior to sequence analysis. A method for the direct cloning and sequence analysis of enzymatically amplified genomic segments has been described by Scharf *et al* (1986). In the method, the primers used in the PCR technique are modified near their 5'-ends to produce convenient restriction sites for cloning directly into, for example, an M13 sequencing victor. After amplification, the PCR products are cleaved with the appropriate restriction enzymes. The restriction fragments are ligated into the M13 vector and transformed into, for example, a JM 103 host, plated out, and the resulting plaques are screened by hybridisation with a labelled oligonucleotide probe. Other methods for cloning and sequence analysis are known in the art.

In a particularly preferred method for use with the present invention, two oligonucleotide primers are synthesised using the partial amino acid sequence and the codon usage patterns from *S. cerevisiae* (Guthrie and Abelson, 1982) as a guide in designing "guessmer" sequences of DNA sequence. These primers are used for PCRs in which cloned yeast genomic library DNA is present as template. The oligonucleotides are synthesised from regions of CDase in which the amino acids show marked codon usage bias and or few degeneracies. The first primer, CDA4R1 (SEQ ID No. 20), is a 42-mer containing 33 nucleotides corresponding to an amino terminus amino acid sequence, while the second, CDA5AS (SEQ ID No. 21), contains nucleotides complementary to sequence located near the carboxy-terminus of the protein.

The sequence of these oligonucleotides is shown in Figure 5. The PCR primers are engineered with *Eco*RI restriction sites at their 5′ ends to facilitate cloning of fragments generated by PCR. A 350 base pair PCR derived fragment is purified by gel electrophoresis and subcloned into an appropriate vector for DNA sequencing.

The PCR-derived fragment may also be used as a CDase specific probe for screening yeast libraries by colony filter hybridisation techniques.

Both the cloned PCR fragments and the genomic clones encoding CDase are subjected to DNA sequence analysis. Variations in the reaction conditions permit analysis of sequences directly adjacent to the primer to several hundred base pairs away. The DNA sequence obtained is shown in Figure 6. As can be seen, the sequences are 93% homologous having 23 mismatches and 330 matches. Figure 7 shows the nucleotide sequence and deduced amino acid sequence of ORF 2, confirming the previously determined amino acid sequence and predicting the addition of only a few amino acids to either end of the partial sequence obtained by analysis of the purified protein.

The DNA sequence data are subsequently used to design new PCR primers for generating amplified DNA cassettes coding for CDase.

The CDase coding sequence can be cloned into any suitable vector or replicon, well known in the art. Examples of recombinant DNA vectors for cloning and host cells which they can transform, in parentheses include the bacteriophage lambda (*E. coli*), pBR322 (*E. coli*), pACYC177 (*E. coli*), pKT230 (gram-negative bacteria), PGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-*E. coli* gram-negative bacteria), pHV14 (*E. coli* and *Bacillus subtilis*), pBD9 (*Bacillus*), pIJ61 (*Streptomyces*), pUC6 (*Streptomyces*), Ylp5 (*Saccharomyces*), YCp19 (*Saccharomyces*) and bovine papilloma virus

(mammalian cells).

In addition to control sequences, it can be desirable to add regulatory sequences that allow for regulation of the expression CDase sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those that cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements can also be present in the vector, for example, enhancer sequences.

An expression vector is constructed so that the CDase coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (ie RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the sequences encoding CDase can be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it can be attached to the control sequences with the appropriate orientation; ie to maintain the reading frame. The control sequences and other regulatory sequences can be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector that already contains the control sequences and an appropriate restriction site.

The coding sequence for the cytosine deaminase is shown in Figure 7.

Example 8: An alternative isolation of the c-*erb*B2 promoter

A 2.0 kb fragment of the c-*erb*B-2 cDNA encoding the extracellular domain of

the putative c-*erb*B-2 protein is excised from the Okayama-Berg cloning vector, pSV7186 (available through Pharmacia, Cat. No. 27-4948-01) using *Nco*I and *Aat*II, blunt-ended using T4 DNA polymerase, and ligated with *Eco*RI linkers (NE Biolabs, Cat. No. 1078). The initial c-*erb*B-2 cDNAs may be derived from patients with adenocarcinoma of the breast. The *Eco*RI-linkered partial c-*erb*B-2 cDNA is then subcloned into *Eco*RI digested pFRSV, an SV40-based derivative of pFR400 (Horwich *et al* 1985). To construct pFRSV, a 2.6 kb *Pvu*II/*Hpa*I fragment is isolated from pKSV10 (commercially available through Pharmacia, Cat. No. 27-4926-01), and blunt-end cloned into *Pvu*II-digested pFR400. The *Bgl*II site at nucleotide position 5107 of pKSV-10 is previously converted to an *Eco*RI site by site-directed mutagenesis (Zoller and Smith 1987) leaving a unique RI cloning site in the final construct, pFRSV. This vector also contains the dominant selectable marker, DHFR, which is utilised for amplification of the gp75 c-*erb*B-2 derivative. The final construct, designated pFRSV-c-*erb*B-2 sec (Figure 8), is transformed into *E. coli* strain, MC1061, and plasmid DNA is isolated according to Sambrook *et al* 1989.

Example 9: A construct containing OB2-1 binding sites upstream of a heterologous promoter

As an additional demonstration of the functional importance of the c-*erb*B-2-binding nuclear transcription activator, we have performed transfection assays with a construct containing several copies of its binding site upstream of a heterologous promoter. We have previously described the use of the reporter plasmid pSS0.2CAT which contains the basal promoter of the human glutathione transferase π gene (GST-π; Cowell *et al*, 1992 *Mol. Cell. Biol.* 12, 3070-3077). We constructed a derivative of this plasmid, pπ213(3)CAT (see below), which contained three copies of the sequence (SEQ ID No. 1) upstream of the basal GST-π promoter. After transfection into the two mammary cell lines, the basal construct was surprisingly active as compared to our control

plasmids. Nevertheless, pπ213(3)CAT showed a 6-fold increase in activity in the overexpressing line, ZR75-1, but no change in activity relative to pSS0.2CAT in the low-expressing line T47D (see Figure 11). Consequently, taking together the results from several different promoter activity assays and the distribution of the -213 binding activity, we propose that the action of this factor on the c-*erb*B-2 promoter contributes to the accumulation of c-*erb*B-2 mRNA in overexpressing breast lines. We have therefore called this factor OB2-1 for overexpression of c-*erb*B-2, factor 1.

Construction of pπ213(3)CAT. The plasmid pπ213(3)CAT was derived from pSS0.02CAT by ligation of three copies of the sequence (SEQ ID No. 1) into the *Hind*III site at -94 within the GST-π promoter. The integrity and orientation of the oligonucleotides (all three in the same orientation as found in the c-*erb*B-2 promoter) were confirmed by sequencing.

Example 10: Promoter region of the MUC1 gene

The mucin gene, MUC1, is selectively expressed in breast and pancreatic cell lines but not in non-epithelial cell lines. The promoter region for this gene may be obtained by the methods taught in WO 91/09867 and is shown in Figure 12.

The 5′ sequences flanking the human MUC1 gene are analyzed for their ability to direct expression of a reporter gene (the chloramphenical transferase gene, CAT) in cell lines which normally express or do not express the MUC1 gene. A construct containing 2.9 kb of MUC1 5′ flanking sequence shows expression of CAT in breast and pancreatic cell lines but not in the non-epithelial cell lines HT 1080, SK23 and HTB96. Deletion analysis shows that maximum expression was obtained in ZR-75 (breast cancer line) and HPAP (pancreatic cancer line) with only 743 bp of 5′ flanking sequence. Sequences within 1.6 kb of the transcriptional start site showed enhancing activity in a vector

carrying an nhancerless SV40 prom ter. Analysis of proximal 5' sequences in a promoterless CAT vector carrying the SV40 enhancer shows that sequences between -60 and -150 were crucial for tissue specific expression. An Spl site at -99/-90 and an E-box (E-MUC1) at -84/-64 in this region are shown by mutational analysis to play a role in the regulation of transcription. Gel shift analysis with oligonucleotides and nuclear extracts of ZR-75 showed protein binding to both of these sites. Spl binding activity is similar in ZR-75 and HT1080 cells whereas binding of factors to the E-MUC1 oligonucleotide reveals quantitative and qualitative differences between epithelial and non-epithelial cells.

The 743 bp of 5' flanking sequence of MUC1 is combined with a tandem array of three OB2-1 binding sites (SEQ ID No. 1) to generate a promoter fragment selective for expression of cytosine deaminase in breast cancer cells.

Further regulatory elements of the MUC1 gene promoter which are useful in the present invention may be identified in Figure 13.

Example 11: Purification of OB2-1, peptide sequencing and antibodies

A diagram of the purification scheme for OB2-1 from ZR751 cells is shown in Figure 14. The key step was the use of a DNA affinity column whereby double-stranded oligonucleotides representing the binding site are immobilised on Sepharose beads. The purified material was concentrated using an Amicon spin concentrator and the sample was run on an SDS-PAGE gel. The gel was stained briefly with Coomassie blue and the two major bands (one at 110kd and one at 46kd) were cut out. These gel slices were treated with trypsin to release peptides which were separated on reverse phase HPLC so that each peptide could be sequenced on an Applied Biosystems automated machine. The 110kd protein proved to be a non-specific DNA binding protein called poly(ADP-

ribose) polymerase (PARP). We obtained antibodies to PARP and showed that they do not disrupt the OB2-1 binding complex in gel shifts, thus indicating that OB2-1 is not PARP. The 46kd species contained peptides highly related to the published sequence of AP2.

FR43: DFAYVCEAEFPSK (SEQ ID No. 6); FR51: AAHVTLLTSLVEGEAVHLAR (SEQ ID No. 7); FR31: IGLNLPAGR (SEQ ID No. 8); FR38: ELVGAVMNPTEVFX (SEQ ID No. 9); FR23: QSQESGLLXTHR (SEQ ID No. 10); FRXX: LSLLSSTSK (SEQ ID No. 11); FR45: DNLFGGVVNPNEVFC (SEQ ID No. 12); FR46: LSPPECLNASLLGGVLR (SEQ ID No. 13); FR47 LSPP (SEQ ID No. 14); FR63: GLPHQQSGL (SEQ ID No. 15); FR39a: ELVEAVMNPS (SEQ ID No. 30); FR39b: ELVGAVMNPS (SEQ ID No. 31); XFLNNTTTNH (SEQ ID No. 32); FR46 top: LSPPECHNASLLGGVLR (SEQ ID No. 33); and XXXLLSHAQXPYSDAPG (SEQ ID No. 34).

OB2-1 antibodies were raised against some of the same purified material which was used for sequencing. Consequently, our antisera contain antibodies against PARP (a ubiquitous, fairly abundant protein) and OB2-1. These antibodies will prevent OB2-1 complex formation, so presumably recognise epitopes within the DNA binding domain of OB2-1.

The OB2-1 antibodies are further purified using an affinity chromatography column wherein an OB2-1 peptide, for example FR43, is covalently bound to a matrix. The OB2-1-specific antibodies are bound to the matrix whereas non-specific antibodies do not bind.

The antibodies are used to detect OB2-1 in enzyme-linked immunoassays (ELISAs) and immunoblotting (Western blotting) of proteins, derived from tumour cells, following separation by polyacrylamide gel electrophoresis.

Example 12: Effect of aurothiomalate (AuTM) on the binding of OB2-1 to its DNA binding site and the effect of AuTM on the expression from the c-*erb*B-2 promoter *in vivo*

A gel shift assay was performed in which equal quantities of highly purified OB2-1 were incubated with a range of concentrations of aurothiomalate (AuTM) for 60 mins on ice before adding the binding-site oligonucleotide probe (see Figure 17). Thiomalic acid (TMA) alone at the highest concentration (and up to 100 μM) had no effect on binding, whereas at a concentration of 50 μM AuTM, binding is abolished. Also, the binding of an unrelated factor (the bZIP protein, ATF1) to its binding site oligo was not affected by AuTM even at 20 μM. Thus, even at low AuTM concentration the binding of OB2-1 to its binding site is effectively blocked. Based on this result we performed a transfection experiment using the c-*erb*B-2 overexpressing line MDA MD453. The plasmids used and the results are shown in Figure 18. The pE4CAT construct just has binding sites for bZIP factors in its promoter and hence would not be expected to be affected by AuTM based on the gel shift assay and Figure 18 shows this to be the case. It is therefore a good control for non-specific cell toxicity. Clearly, addition of AuTM to the tissue culture medium after transfection only affects the p300CAT construct which contains the OB2-1 binding site and not pE4CAT nor, pCAT basic which lacks the OB2-1 binding site.

Example 13: An *in vivo* assay for compounds which inhibit OB2-1 function

A DNA construct is made by replacing the CAT coding sequence of plasmid pπ213(3)CAT (as described in Example 9) with the secretable placental alkaline phosphatase (SEAP) coding sequence (as described by Berger *et al* (1988) *Gene* **66**, 1-10; incorporated herein by reference). This DNA construct is stably transfected into the c-*erbB*-2 overexpressing breast cell line ZR75-1 to give

"OB2-1" cells. A control cell line is established by stably transfecting ZR75-1 with an essentially equivalent construct but without the OB2-1 binding sites in the GSTπ promoter. This gives "control" cells. Cultures of the "OB2-1" cells and "control" cells are set up in 96 well cell culture plates and the test chemicals are added to the cell cultures. The alkaline phosphatase substrate PNPP is added to 12 mM final concentration. The cultures are quenched with sodium hydroxide (between 0.1M and 1.0M) and the absorbance of the supernatant is measured at 405 nm to indicate the presence of *p*-nitrophenol.

One convenient way of assaying for alkaline phosphatase is to remove a 250 μl aliquot of medium from the transfected plate and heated at 65°C for 5 min. The medium is then clarified by centrifugation in a microfuge at 14000 x *g* for 2 min. An aliquot of medium (10 or 100 μl) was then adjusted to 1 x SEAP assay buffer (1.0M diethanolamine pH 9.8, 0.5 mM $MgCl_2$, 10 mM L-homoarginine) in a final volume of 200 μl and prewarmed to 37°C for 10 min in a 96-well flat-bottom culture dish (Corning). Twenty μl of pre-warmed 120 mM *p*-nitrophenylphosphate dissolved in SEAP assay buffer was then added with mixing. The A_{405} of the reaction mixture was read in an Artek automatic plate reader at 1-min intervals. The change in absorbance was plotted and the maximum linear reaction rate determined. The heating step and the inclusion of L-homoarginine in the assay buffer inhibit any endogenous phosphatase activities.

A comparison is made between the level of *p*-nitrophenol present in the "OB2-1" cells and the "control" cells. Compounds which consistently lead to a lowering of the level of expression (as indicated by a lower absorbance at 405 nm) in the "OB2-1" cells but have minimal effect on expression levels in "control" cells are investigated further.

Aurothiomalate is used as a positive control as disclosed in Example 12.

As an alternative to using PNPP as the substrate for alkaline phosphatase, D-luciferin-O-phosphate can be used as described by Miska & Geiger (1987) *J. Clin. Chem. Clin. Biochem.*, 23-30.

Example 14: Treatment of metastatic breast cancer

Prior to treating a patient an excision biopsy of one nodule is performed together with fine needle aspirates (FNA) of at least five other nodules. The biopsy is examined histologically to confirm metastatic breast cancer. Antibody staining to confirm increased *ERBB2* or OB2-1 expression is performed. A Southern blot to determine gene copy number of *ERBB2* is also carried out.

Dosage schedule

c-*erb*B-2:CDase plasmid

A 22 gauge needle is used to transfer a dose of recombinant DNA in 0.2 ml of Ringer's lactate into the centre of one tumour nodule. Prior to injection, with the needle in place, gentle aspiration is applied to the syringe to ensure that no material is injected intravenously. A dose escalation from 20 μg to 400 μg per injection is used (20, 50, 100, 300, 300 and 400 μg). If tolerated satisfactorily the highest dose will be used subsequently.

5-Fluorocytosine

An intravenous bolus dose of 5-fluorocytosine (*Alcobon*, Roche) is given for three times daily days, 48 hours following the intratumoural injection of DNA. A dose of 300 mg/kg is administered in each 24 hour period in normal saline. In the majority of patients this will achieve a peak plasma concentration of over 200 mg/l. This is the standard dose administered to patients with serious

systemic yeast infections. Possible side effects include nausea, vomiting, diarrhoea, rashes, thrombocytopenia and leucopenia. These are monitored and if necessary the dose reduced accordingly.

5

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CLAIMS

1. The human c-*erb*B-2 promoter-binding nuclear transcription activator OB2-1 or a fragment or variant thereof immunologically cross-reactive therewith.

2. A polypeptide immunologically reactive with OB2-1.

3. A nucleic acid encoding the activator, fragment or variant of Claim 1.

4. A method to aid prognosis or diagnosis for a cancer patient or to determine their suitability for treatment comprising the steps (1) removing a sample of tumour tissue from the patient, (2) measuring the amount of OB2-1 or AP-2 present in the sample, (3) measuring the amount of a marker compound in the sample, (4) computing a ratio of OB2-1 or AP-2 to marker compound, and (5) comparing the said ratio with a ratio computed in the same manner for non-tumour tissue.

5. A method according to Claim 4 wherein the amount of OB2-1 is measured using the polypeptide of Claim 2.

6. A method according to Claim 4 wherein the amount of AP-2 is measured using a polypeptide immunologically reactive with AP-2.

7. A method according to Claim 6 wherein the said immunologically reactive polypeptide reacts with the region of AP-2 between amino acid 295 and amino acid 437.

8. A method according to Claim 4 wherein the amount of OB2-1 is measured by determining the amount of OB2-1 or AP-2 mRNA present.

9. A method of Claim 4 wherein the tumour tissue is breast tumour tissue.

10. Use of a polypeptide immunologically reactive with OB2-1 or AP-2 in the manufacture of a reagent for diagnosis or prognosis of cancer in a patient or for determining their suitability for treatment.

11. A method of predicting whether a patient would be more, or less, likely to benefit from the treatment with the medicaments of Claims 20 to 28 or compositions of Claims 45 to 53 or auranofin, the method of predicting comprising the method of Claim 4.

12. Use of a polypeptide immunologically reactive with OB2-1 or AP-2 in the manufacture of a reagent for predicting whether a patient would be more, or less, likely to benefit from the treatment with the medicaments of Claims 20 to 28 or compositions of Claims 45 to 53 or auranofin.

13. An assay for a compound which reduces the binding of OB2-1 to its DNA binding site, the assay comprising the steps (1) adding the compound to purified OB2-1; (2) further adding an OB2-1 binding site; and (3) determining whether OB2-1 is bound to the OB2-1 binding site.

14. An assay for a compound which reduces the activity of OB2-1,

the assay comprising the steps (1) transfecting a cell with an active promoter comprising an OB2-1 binding site which promoter drives the expression of a reporter gene; (2) adding to the said cell or a product of the said cell the compound; and (3) determining whether the activity of the said promoter is reduced.

15. An assay according to Claim 14 wherein the reporter gene encodes an enzyme which can be readily assayed.

16. An assay according to Claim 15 wherein the enzyme is alkaline phosphatase.

17. An assay according to Claim 16 wherein the alkaline phosphatase is secreted from said cell.

18. A compound identified by the assay of Claim 13.

19. A compound identified by the assay of Claim 14.

20. Use of a compound that reduces the binding of OB2-1 or AP-2 to its DNA binding site in the manufacture of a medicament for treating cancer in a patient.

21. Use according to Claim 20 wherein the compound has the general formula:

Au-X-R

wherein Au is gold, X is a linking group and R is a water soluble moiety.

22. Use according to Claim 21 wherein X is S and R is a sugar

moiety.

23. Use according to Claim 21 wherein X is S and R is an organic acid.

24. Use according to Claim 22 wherein the compound is aurothioglucose.

25. Use according to Claim 23 wherein the compound is aurothiomalic acid or a pharmaceutically acceptable salt thereof.

26. Use according to Claim 20 wherein the compound is selected from the group consisting of pharmaceutically acceptable compositions of Zn^{2+} or Cd^{2+} and aromatic C-nitroso compounds.

27. Use according to Claim 20 wherein the compound is a compound of Claim 18 or 19.

28. Use according to Claim 20 wherein the cancer is breast cancer.

29. A method of treating cancer in a patient comprising administering to the patient a tumour-inhibiting amount of a compound that reduces the binding of OB2-1 or AP-2 to its DNA binding site.

30. A DNA construct comprising (i) a promoter segment which specifically binds the c-*erb*B-2-binding nuclear transcription activator (OB2-1) and, under transcriptional regulation thereby, (ii) a heterologous coding sequence.

31. A construct according to Claim 30 wherein the said segment

comprises the c-*erb*B-2 promoter or a fragment thereof.

32. A construct according to Claim 30 wherein the said segment comprises the c-*erb*B-3 promoter or a fragment thereof.

33. A construct according to Claim 30 wherein the segment includes the sequence (SEQ ID No. 1)

```
-226 GAGAACGGCTGCAGGCAACCCAGGCGT -200
     CTCTTGCCGACGTCCGTTGGGTCCGCA
```

34. A construct according to Claim 30 wherein the segment includes the sequence

GCCTCTGGC
CGGAGACCG (SEQ ID No. 4).

35. A construct according to Claim 30 wherein the segment comprises at least one binding site for the OB2-1 protein.

36. A construct according to Claim 35 wherein the segment further comprises a segment from an epithelial cell specific promoter which segment confers cell-type selectivity.

37. A construct according to Claim 36 wherein the epithelial cell specific promoter is the MUC1 gene promoter.

38. A construct according to Claim 37 wherein the segment which confers cell-type selectivity is selected from the 743 bp immediately upstream of the transcriptional initiation site of the MUC1 gene.

39. A construct according to Claim 35 wherein the segment further comprises a segment from a mammary cell specific promoter which segment confers cell-type selectivity.

40. A construct according to Claim 39 wherein the mammary cell specific promoter is the MMTV LTR promoter.

41. A construct according to Claim 39 wherein the segment which confers cell-type selectivity includes a MAF-binding sequence.

42. A construct according to Claim 30 wherein the heterologous coding sequence encodes a directly or indirectly cytotoxic compound.

43. A construct according to Claim 42 wherein the said compound is an enzyme capable of converting a non-toxic pro-drug into a cytotoxic drug.

44. A construct according to Claim 43 wherein the enzyme converts 5-fluorocytosine to 5-fluoro-uracil.

45. A composition comprising a construct according to Claim 30 and means to deliver the construct into a mammalian cell for expression therein of the heterologous coding sequence.

46. A composition according to Claim 45 wherein the composition is a pharmaceutical composition and the said mammalian cell is a cell in a human patient.

47. A composition according to Claim 46 wherein the means to

deliver the construct comprises a liposome.

48. A composition according to Claim 46 wherein the means to deliver the construct comprises transferrin, or a fragment thereof, that binds the transferrin receptor.

49. A composition according to Claim 46 wherein the means to deliver the construct comprises inactivated adenovirus.

50. A composition according to Claim 46 wherein the means to deliver the construct comprises a retrovirus.

51. A composition according to Claim 46 wherein the composition provides for specific delivery of the construct to tumour cells.

52. A composition according to Claim 51 wherein the provision for specific delivery comprises an antibody, or fragment thereof, reactive towards a tumour cell surface antigen.

53. A composition according to Claim 51 wherein the provision for specific delivery comprises a ligand for the c-*erb*B-2 receptor.

54. Use of a composition according to Claim 46 in the manufacture of a medicament for treating cancer in a patient.

55. Use according to Claim 54 wherein the heterologous coding sequence encodes an enzyme capable of converting a relatively non-toxic pro-drug into a cytotoxic drug, and the relatively non-toxic pro-drug is administered concomitantly with the composition.

56. Use according to Claim 55 wherein the heterologous coding sequence encodes an enzyme which converts 5-fluorocytosine to 5-fluorouracil and the relatively non-toxic pro-drug is 5-fluorocytosine.

57. A method of treating cancer in a patient comprising administering to the patient a tumour-inhibiting, relatively non-toxic amount of a composition according to Claim 46.

58. A method of treating cancer in a patient comprising (1) determining their suitability for treatment using the method steps defined in Claim 4 and, if the patients are suitable for treatment (2) administering a tumour-inhibitory amount of a compound as defined in any one of Claims 18 to 26 or Claims 45 to 53 or auranofin.

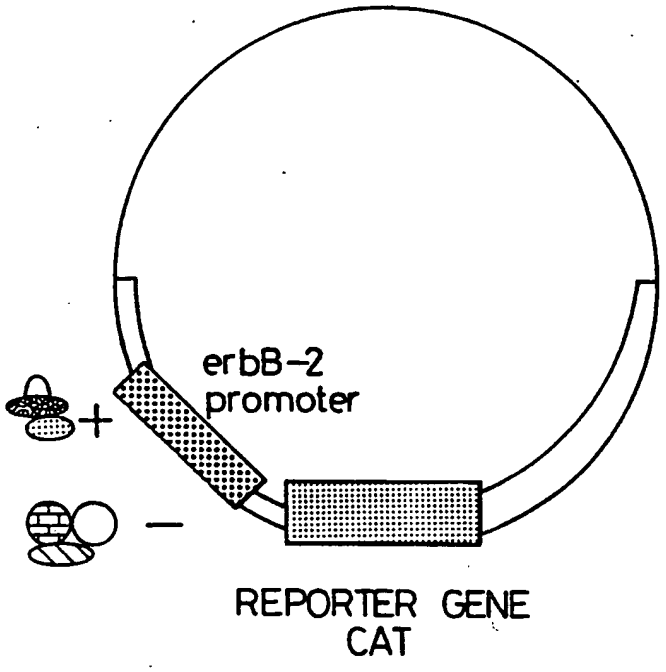

erbB-2
promoter
+
−
REPORTER GENE
CAT

Fig. 1

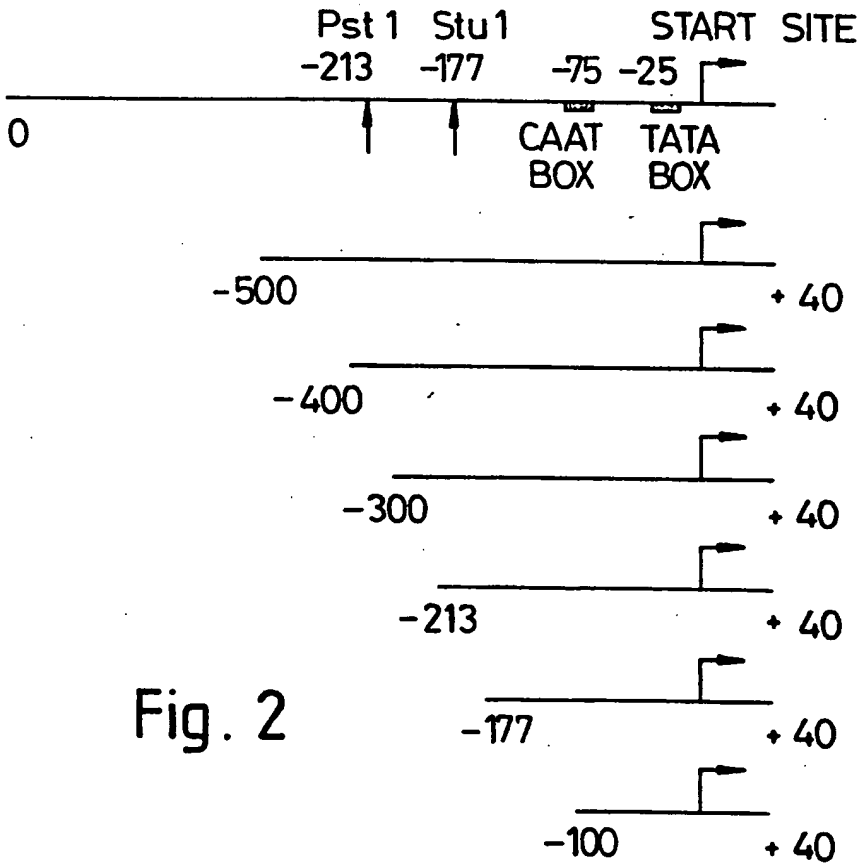

Pst 1
Stu 1
START SITE
-213
-177
-75
-25
0
CAAT
BOX
TATA
BOX
-500
+ 40
-400
+ 40
-300
+ 40
-213
+ 40
-177
+ 40
-100
+ 40

Fig. 2

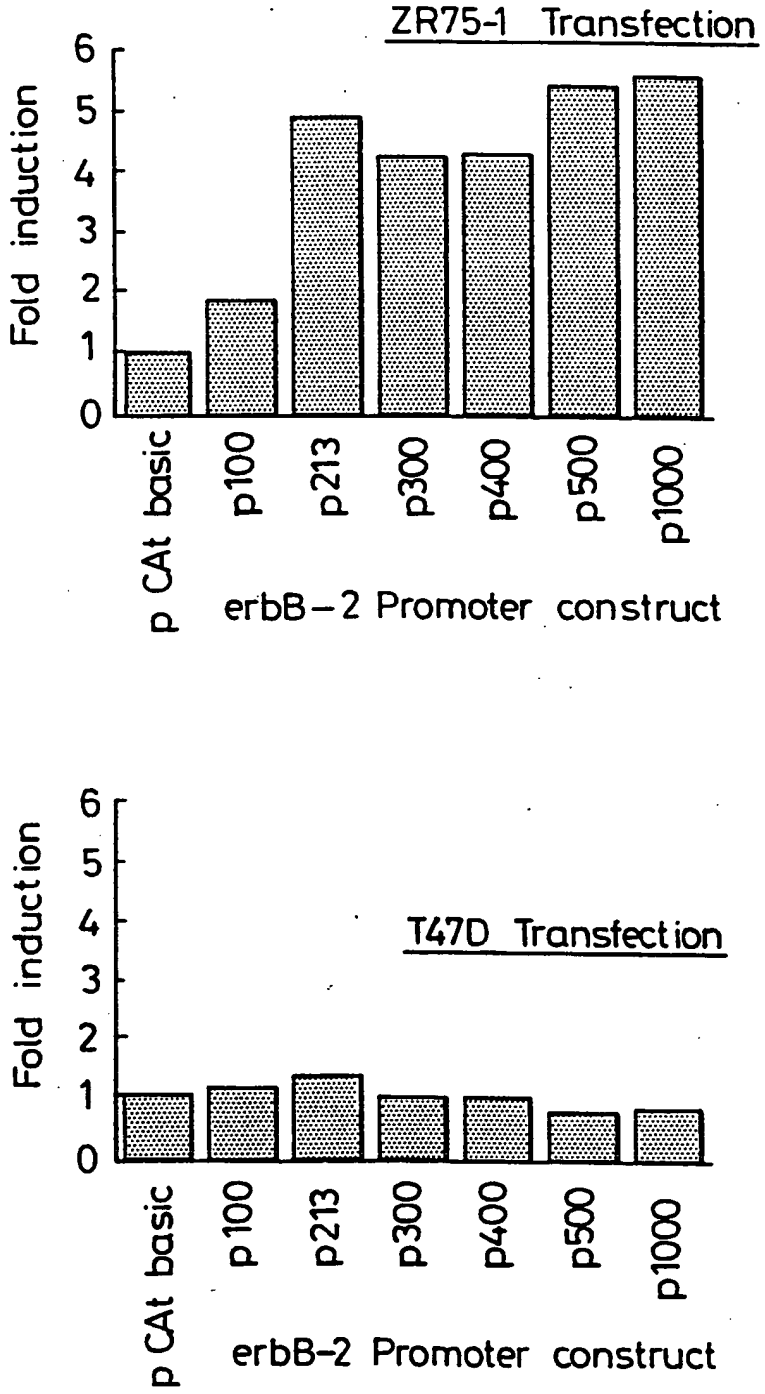

ZR75-1 Transfection
Fold induction
0
1
2
3
4
5
6
p CAt basic
p100
p213
p300
p400
p500
p1000
erbB-2 Promoter construct
T47D Transfection
Fold induction
0
1
2
3
4
5
6
p CAt basic
p100
p213
p300
p400
p500
p1000
erbB-2 Promoter construct

Fig. 3

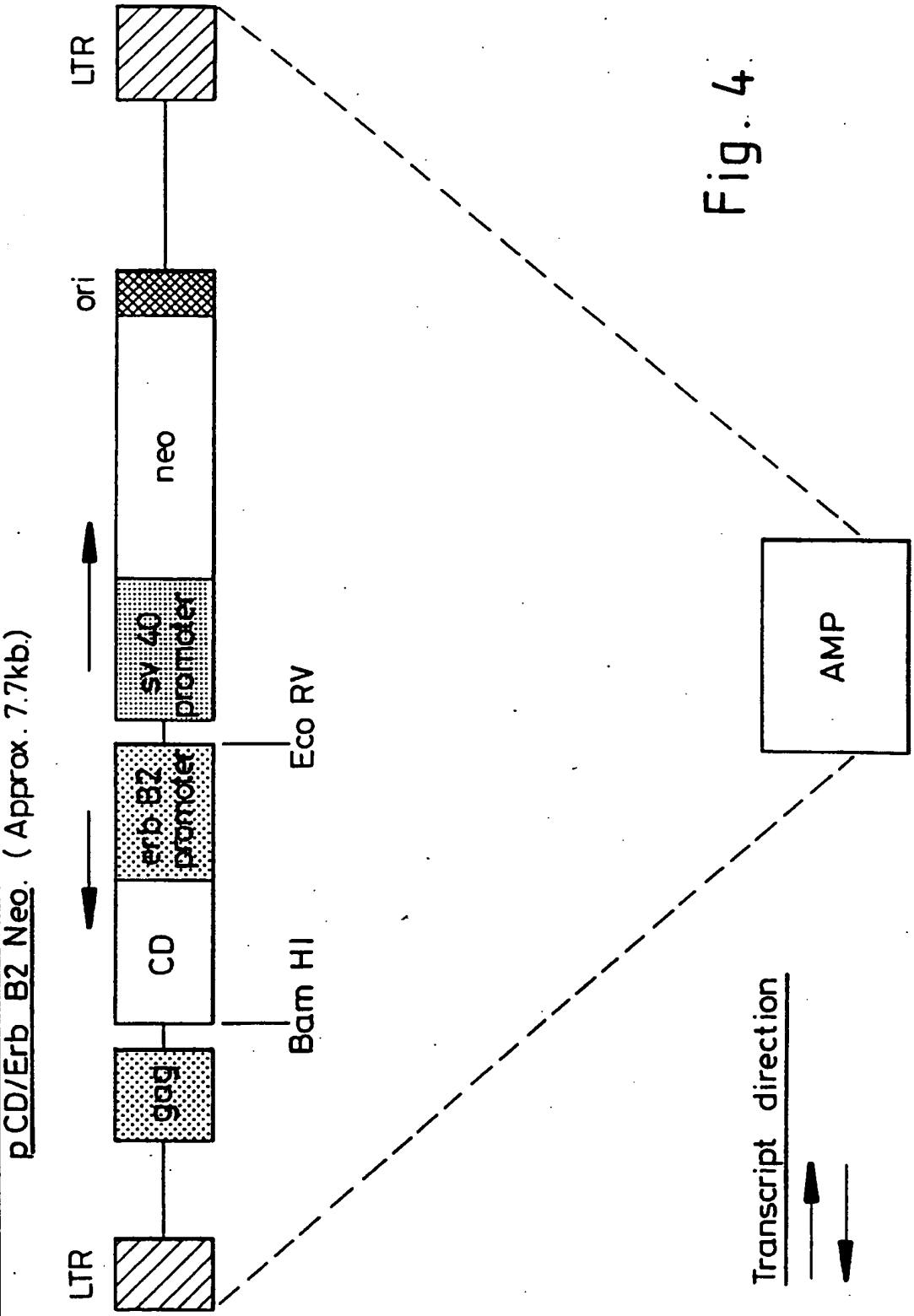

LTR
ori
neo
sv 40 promoter
erb B2 promoter
CD
gag
LTR
Eco RV
Bam HI
AMP
Transcript direction
pCD/Erb B2 Neo. (Approx. 7.7kb.)

Fig. 4

```
1
M   V   T   G   G   M   A   S   K   W   D   Q   K   G   M

D   I   A   Y   E   E   A   A   L   G   Y   K   E   G   G

V   P   I   G   G   C   L   I   N   N   K   D   G   S   V

PRIMER CDA4/RI:      (5'GGGGAATTC--)
    GGT AGA GGT CAC AAC ATG CGT TTC CAA AAG GGT 3'  --->
L   G   R   G   H   N   M   R   F   Q   K   G   S   A   T
    47

L   H   G   E   I   S   T   L   E   N   C   G   R   L   E

G   K   V   Y   K   D   T   T   L   Y   T   T   L   S   P

C   D   M   C   T   G   A   I   I   M   Y   G   I   P   R

C   V   V   G   E   N   V   N   F   K   S   K   G   E   K

Y   L   Q   T   R   G   H   E   V   V   V   V   D   D   E

                                                147
R   C   K   K   I   M   K   Q   F   I   D   E   R   P   Q
                                          <---3'CTT TCT GGT GTT

                        157 158
D   W   F   E   D   I   G   E
CTA ACC AAG CTT CTG TAG CCA
  G               A   A     (--CTTAAGGGG 5') PRIMER CDA AS5'
```

Figure 5

```
TTCTCCTCAT ATCACGTGTC ATTCTGCAGG GCGGTAGTAC CGAGACCCTG ACTTTCTTTT      60
TTTTTTGCGA AATTAAAAAG TTCATTTTCA ATTCGACAAT GAGATCTACA AGCCATTGTT     120
TTATGTTGAT GAGAGCCAGC TTAAAGAGTT AAAAATTTCA TAGCTAATGG TGACAGGGGG     180
AATGGCAAGC AAGTGGGATC AGAAGGGTAT GGACATTGCC TATGAGGAGG CGGCCTTAGG     240
TTACAAAGAG GGTGGTGTTC CTATTGGCGG ATGTCTTATC AATAACAAAG ACGGAAGTGT     300
TCTCGGTCGT GGTCACAACA TGAGATTTCA AAAGGGTTCC GCCACACTAC ATGGTGAGAT     360
CTCCACTTTG GAAAACTGTG GGAGATTAGA GGGCAAAGTG TACAAAGATA CCACTTTGTA     420
TACGACGCTG TCTCCATGCG ACATGTGTAC AGGTGCCATC ATCATGTATG GTATTCCACG     480
CTGTGTTGTC GGTGAGAACG TTAATTTCAA AAGTAAGGGC GAGAAATATT TACAAACTAG     540
AGGTCACGAG GTTGTTGTTG TTGACGATGA GAGGTGTAAA AAGATCATGA AACAATTTAT     600
CGATGAAAGA CCTCAGGATT GGTTTGAAGA TATTGGTGAG TAGAGCACGC AGCACGCTGT     660
ATTTACGTAT TTAATTTTAT ATATTTGTGC ATACACTACT AGGGAAGACT TGAAAAAAAC     720
CTAGGAAATG AAAAAACGAC ACAGGAAGTC CCGTATTTAC TATTTTTTCC TTCCTTTTGA     780
TGGGGCAGGG CGGAAATAGA GGATAGGATA AGCCATCTGC TTAGCTGTTT CCGTCTCATC     840
TTCGGTAGTT GTCTCATGTC GTTCAGTATA CTTAGAGCGC AT                        882
```

Figure 6

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    START
ATG GTG ACA GGG GGA ATG GCA AGC AAG TGG GAT CAG AAG GGT ATG   211
M   V   T   G   G   M   A   S   K   W   D   Q   K   G   M

GAC ATT GCC TAT GAG GAG GCG GCC TTA GGT TAC AAA GAG GGT GGT   256
D   I   A   Y   E   E   A   A   L   G   Y   K   E   G   G

GTT CCT ATT GGC GGA TGT CTT ATC AAT AAC AAA GAC GGA AGT GTT   301
V   P   I   G   G   C   L   I   N   N   K   D   G   S   V

CTC GGT CGT GGT CAC AAC ATG AGA TTT CAA AAG GGT TCC GCC ACA   346
L   G   R   G   H   N   M   R   F   Q   K   G   S   A   T

CTA CAT GGT GAG ATC TCC ACT TTG GAA AAC TGT GGG AGA TTA GAG   391
L   H   G   E   I   S   T   L   E   N   C   G   R   L   E

GGC AAA GTG TAC AAA GAT ACC ACT TTG TAT ACG ACG CTG TCT CCA   436
G   K   V   Y   K   D   T   T   L   Y   T   T   L   S   P

TGC GAC ATG TGT ACA GGT GCC ATC ATC ATG TAT GGT ATT CCA CGC   481
C   D   M   C   T   G   A   I   I   M   Y   G   I   P   R

TGT GTT GTC GGT GAG AAC GTT AAT TTC AAA AGT AAG GGC GAG AAA   526
C   V   V   G   E   N   V   N   F   K   S   K   G   E   K

TAT TTA CAA ACT AGA GGT CAC GAG GTT GTT GTT GTT GAC GAT GAG   571
Y   L   Q   T   R   G   H   E   V   V   V   V   D   D   E

AGG TGT AAA AAG ATC ATG AAA CAA TTT ATC GAT GAA AGA CCT CAG   616
R   C   K   K   I   M   K   Q   F   I   D   E   R   P   Q

GAT TGG TTT GAA GAT ATT GGT GAG TAG                          641
D   W   F   E   D   I   G   E   STOP
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Figure 7

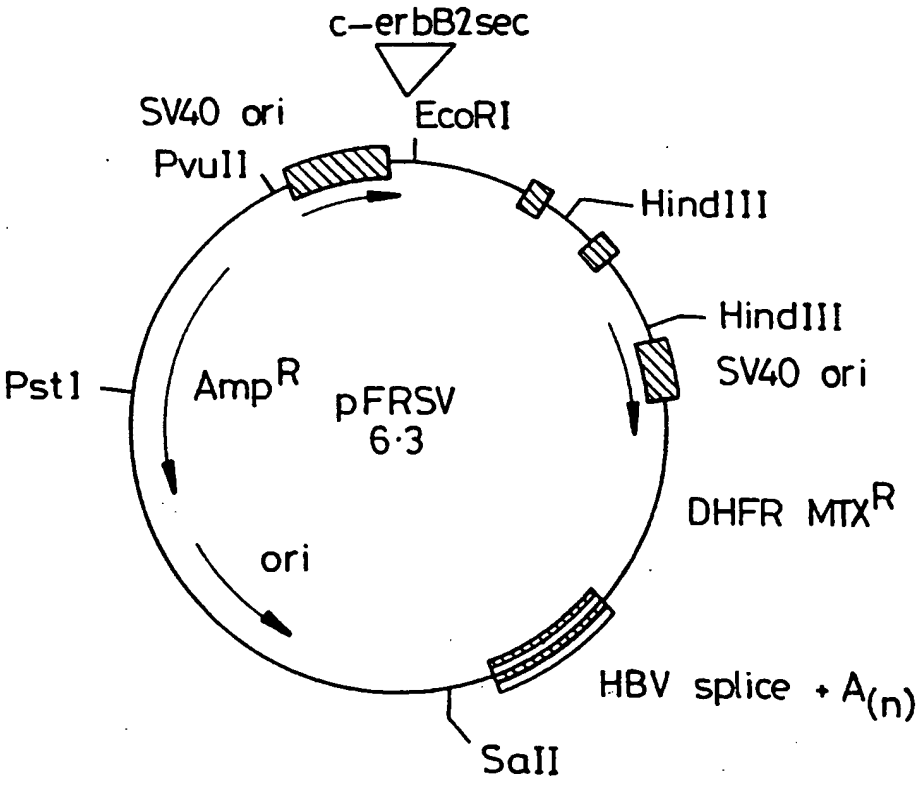

c-erbB2sec
SV40 ori
PvuII
EcoRI
HindIII
HindIII
SV40 ori
PstI
Amp^R
pFRSV
6·3
DHFR MTX^R
ori
HBV splice + A$_{(n)}$
SalI

Fig. 8

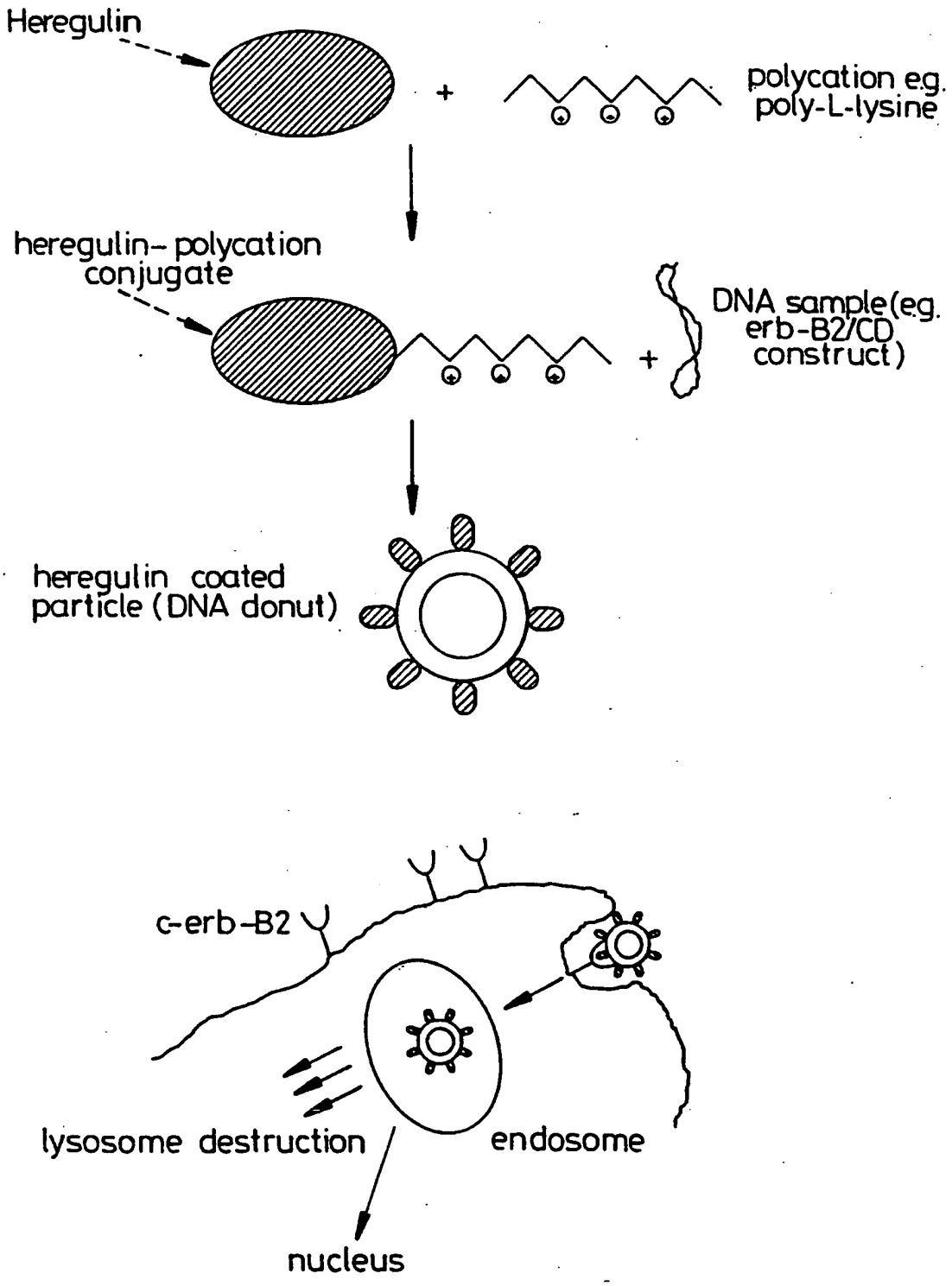

Heregulin
+
polycation eg.
poly-L-lysine
heregulin-polycation
conjugate
+
DNA sample(eg.
erb-B2/CD
construct)
heregulin coated
particle (DNA donut)
c-erb-B2
lysosome destruction
endosome
nucleus

Fig. 9

```
GAATTCAGGC TAGCA ATG TCG AAT AAC GCT TTA CAA ACA ATT ATT AAC GCC          51
                 Met Ser Asn Asn Ala Leu Gln Thr Ile Ile Asn Ala
                   1               5                  10

CGG TTA CCA GGC GAA GAG GGG CTG TGG CAG ATT CAT CTG CAG GAC GGA          99
Arg Leu Pro Gly Glu Glu Gly Leu Trp Gln Ile His Leu Gln Asp Gly
         15                  20                  25

AAA ATC AGC GCC ATT GAT GCG CAA TCC GGC GTG ATG CCC ATA ACT GAA         147
Lys Ile Ser Ala Ile Asp Ala Gln Ser Gly Val Met Pro Ile Thr Glu
     30                  35                  40

AAC AGC CTG GAT GCC GAA CAA GGT TTA GTT ATA CCG CCG TTT GTG GAG         195
Asn Ser Leu Asp Ala Glu Gln Gly Leu Val Ile Pro Pro Phe Val Glu
 45                  50                  55                  60

CCA CAT ATT CAC CTG GAC ACC ACG CAA ACC GCC GGA CAA CCG AAC TGG         243
Pro His Ile His Leu Asp Thr Thr Gln Thr Ala Gly Gln Pro Asn Trp
                 65                  70                  75

AAT CAG TCC GGC ACG CTG TTT GAA GGC ATT GAA CGC TGG GCC GAG CGC         291
Asn Gln Ser Gly Thr Leu Phe Glu Gly Ile Glu Arg Trp Ala Glu Arg
             80                  85                  90

AAA GCG TTA TTA ACC CAT GAC GAT GTG AAA CAA CGC GCA TGG CAA ACG         339
Lys Ala Leu Leu Thr His Asp Asp Val Lys Gln Arg Ala Trp Gln Thr
         95                  100                 105

CTG AAA TGG CAG ATT GCC AAC GGC ATT CAG CAT GTG CGT ACC CAT GTC         387
Leu Lys Trp Gln Ile Ala Asn Gly Ile Gln His Val Arg Thr His Val
     110                 115                 120

GAT GTT TCG GAT GCA ACG CTA ACT GCG CTG AAA GCA ATG CTG GAA GTG         435
Asp Val Ser Asp Ala Thr Leu Thr Ala Leu Lys Ala Met Leu Glu Val
 125                 130                 135                 140

AAG CAG GAA GTC GCG CCG TGG ATT GAT CTG CAA ATC GTC GCC TTC CCT         483
Lys Gln Glu Val Ala Pro Trp Ile Asp Leu Gln Ile Val Ala Phe Pro
                 145                 150                 155

CAG GAA GGG ATT TTG TCG TAT CCC AAC GGT GAA GCG TTG CTG GAA GAG         531
Gln Glu Gly Ile Leu Ser Tyr Pro Asn Gly Glu Ala Leu Leu Glu Glu
             160                 165                 170

GCG TTA CGC TTA GGG GCA GAT GTA GTG GGG GCG ATT CCG CAT TTT GAA         579
Ala Leu Arg Leu Gly Ala Asp Val Val Gly Ala Ile Pro His Phe Glu
         175                 180                 185

TTT ACC CGT GAA TAC GGC GTG GAG TCG CTG CAT AAA ACC TTC GCC CTG         627
Phe Thr Arg Glu Tyr Gly Val Glu Ser Leu His Lys Thr Phe Ala Leu
     190                 195                 200

GCG CAA AAA TAC GAC CGT CTC ATC GAC GTT CAC TGT GAT GAG ATC GAT         675
Ala Gln Lys Tyr Asp Arg Leu Ile Asp Val His Cys Asp Glu Ile Asp
 205                 210                 215                 220

GAC GAG CAG TCG CGC TTT GTC GAA ACC GTT GCT GCC CTG GCG CAC CAT         723
Asp Glu Gln Ser Arg Phe Val Glu Thr Val Ala Ala Leu Ala His His
                 225                 230                 235

GAA GGC ATG GGC GCG CGA GTC ACC GCC AGC CAC ACC ACG GCA ATG CAC         771
Glu Gly Met Gly Ala Arg Val Thr Ala Ser His Thr Thr Ala Met His
             240                 245                 250

TCC TAT AAC GGG GCG TAT ACC TCA CGC CTG TTC CGC TTG CTG AAA ATG         819
Ser Tyr Asn Gly Ala Tyr Thr Ser Arg Leu Phe Arg Leu Leu Lys Met
         255                 260                 265
```

Figure 10 Part 1 of 2

```
TCC GGT ATT AAC TTT GTC GCC AAC CCG CTG GTC AAT ATT CAT CTG CAA      867
Ser Gly Ile Asn Phe Val Ala Asn Pro Leu Val Asn Ile His Leu Gln
    270                 275                 280

GGA CGT TTC GAT ACG TAT CCA AAA CGT CGC GGC ATC ACG CGC GTT AAA      915
Gly Arg Phe Asp Thr Tyr Pro Lys Arg Arg Gly Ile Thr Arg Val Lys
285                 290                 295                 300

GAG ATG CTG GAG TCC GGC ATT AAC GTC TGC TTT GGT CAC GAT GAT GTC      963
Glu Met Leu Glu Ser Gly Ile Asn Val Cys Phe Gly His Asp Asp Val
                305                 310                 315

TTC GAT CCG TGG TAT CCG CTG GGA ACG GCG AAT ATG CTG CAA GTG CTG     1011
Phe Asp Pro Trp Tyr Pro Leu Gly Thr Ala Asn Met Leu Gln Val Leu
            320                 325                 330

CAT ATG GGG CTG CAT GTT TGC CAG TTG ATG GGC TAC GGG CAG ATT AAC     1059
His Met Gly Leu His Val Cys Gln Leu Met Gly Tyr Gly Gln Ile Asn
        335                 340                 345

GAT GGC CTG AAT TTA ATC ACC CAC CAC AGC GCA AGG ACG TTG AAT TTG     1107
Asp Gly Leu Asn Leu Ile Thr His His Ser Ala Arg Thr Leu Asn Leu
    350                 355                 360

CAG GAT TAC GGC ATT GCC GCC GGA AAC AGC GCC AAC CTG ATT ATC CTG     1155
Gln Asp Tyr Gly Ile Ala Ala Gly Asn Ser Ala Asn Leu Ile Ile Leu
365                 370                 375                 380

CCG GCT GAA AAT GGG TTT GAT GCG CTG CGC CGT CAG GTT CCG GTA CGT     1203
Pro Ala Glu Asn Gly Phe Asp Ala Leu Arg Arg Gln Val Pro Val Arg
                385                 390                 395

TAT TCG GTA CGT GGC GGC AAG GTG ATT GCC AGC ACA CAA CCG GCA CAA     1251
Tyr Ser Val Arg Gly Gly Lys Val Ile Ala Ser Thr Gln Pro Ala Gln
            400                 405                 410

ACC ACC GTA TAT CTG GAG CAG CCA GAA GCC ATC GAT TAC AAA CGT        1296
Thr Thr Val Tyr Leu Glu Gln Pro Glu Ala Ile Asp Tyr Lys Arg
        415                 420                 425

TGAACGACTG GGTTACAGCG AGCTTAGTTT ATGCCGGATG CGCGGTGAAC GCCTTATCCG   1356

GCCTACGTAG AGCACTGAAC TCGTAGGCCT GATAAGCGTA GCGCATCAGG CAATTCCAGC   1416

CGCTGATCTG TGTCAGCGGC TACCGTGATT CATTCCCGCC AACAACCGCG CATTCCTCCA   1476

ACGCCATGTG CAAAAATGCC TTCGCAGCGG CTGTCTGCCA GCTAGAG                 1523
```

Figure 10 Part 2 of 2

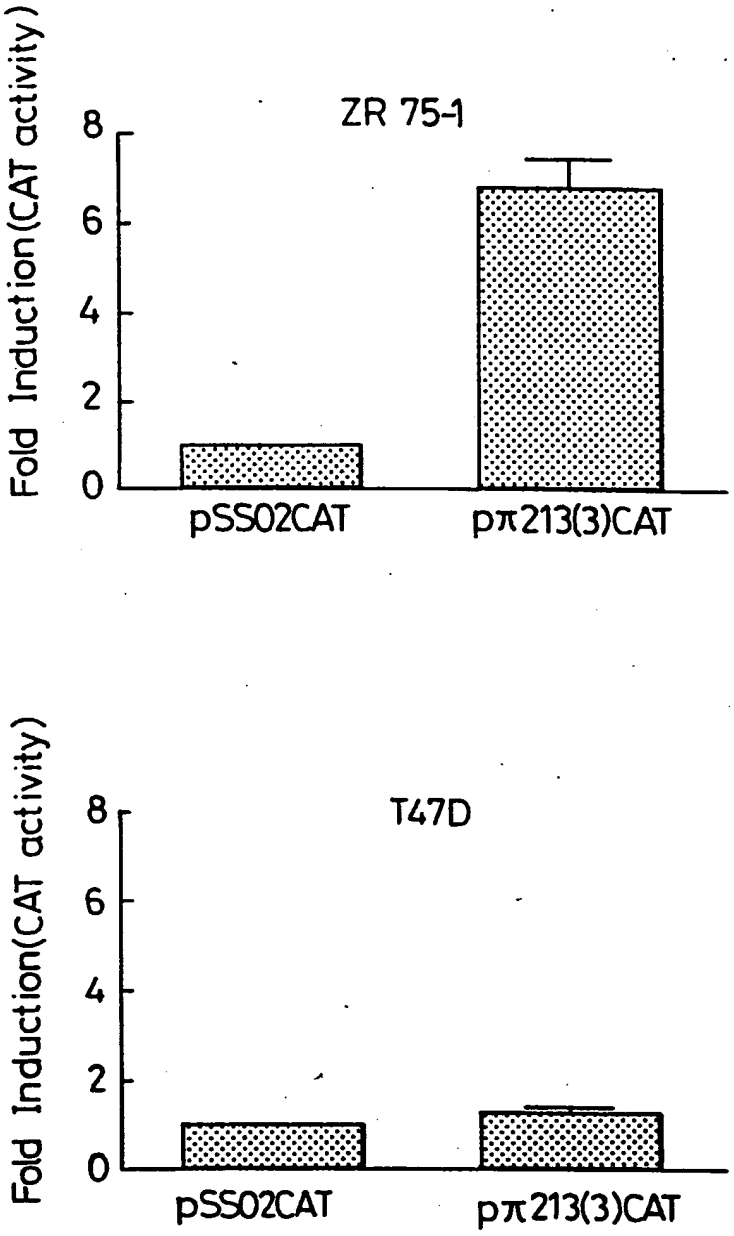

Fold Induction(CAT activity)
ZR 75-1
8
6
4
2
0
pSS02CAT
pπ213(3)CAT
Fold Induction(CAT activity)
T47D
8
6
4
2
0
pSS02CAT
pπ213(3)CAT

Fig. 11

SUBSTITUTE SHEET (RULE 26)

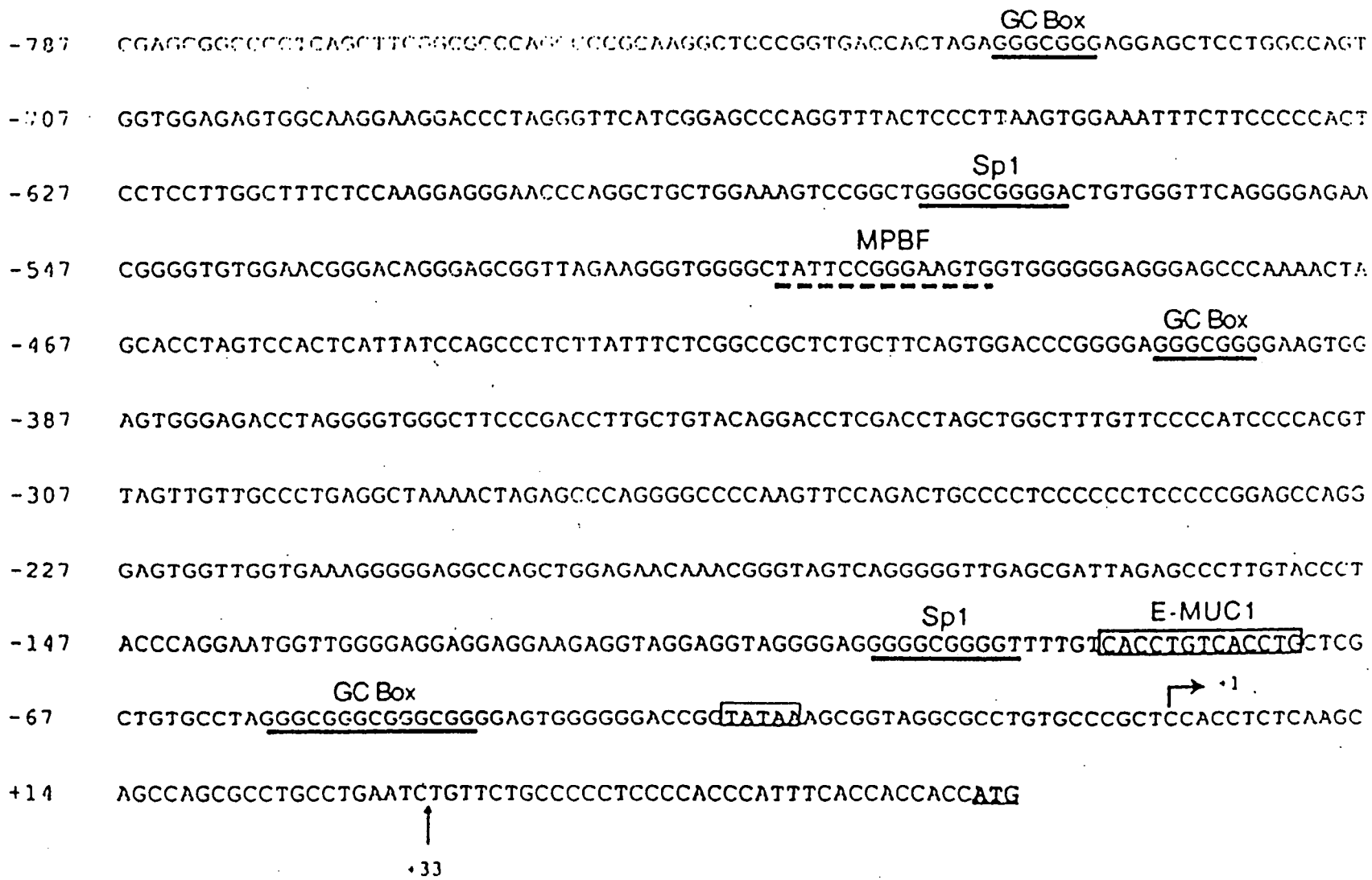


Figure 12

SUBSTITUTE SHEET (RULE 26)

Regulatory element	Consensus Sequence+	Sequence in PEM	Location
SP1	GGGCGG	GGGCGG	-727
		GGGCGG	-397
		GGGCGG	-94
		GGGCGGGCGGGCGGG	-54
SV40 enhancer element			
a	ATGTGTGT	CTGTGGGT	-562
b	GCATGCAT	GCCTGCCT	+25
c	GTGGATAG	GTGGAGAG	-702
AP-1	CTGACTCA	GTGACCAC	-739
	G A	CTGCTTCA	-418
		GTGCCTAG	-61
		CTGCCTGA	+27
AP-2	CCCCAGGC	ACCCAGGC	-597
	G G	CACCGGGC	+77
NF1/CTF	TTGGCTNNNAGCCAA	TTGGCTTTCTCCAA	-618
Glucocorticoid regulatory element:			
Core sequence	TGTTCT	TGTTCT	+38
		TGTTCC	-321
Consensus sequence	GGTACANNNTGTTCT	GCCTGAATCTGTTCT	+29
		AGCTGGCTTTGTTCC	-330

Figure 13 Part 1 of 2

CACCC factor	CACCC	CACCC CACCC	+54 +84
Progesterone receptor consensus sequence	ATTCCTCTGT	ACTCCTCTCC ACTCCTCCTT ATTTCTCGGC	-802 -626 -432
Estrogen consensus sequence	GGTCANNNTGACC	GCTCCCGGTGACC	-746
RNA Polymerase III			
Box A	RRYNNARYXGG	GACCTAGCTGG AGTGGAGTGGG	-335 -388
Box B	GWTCRANNC	GTTCCAGAC	-260
Enhancer sequences:			
Interferon-ß seq	GGAAATTCCTCTG	GGAAATTTCTTCC	-642
CMV enhancer	GGAAAGTCCCGTT	GGAAAGTCCGGCT	-585

Figure 13 Part 2 of 2

SUBSTITUTE SHEET (RULE 26)

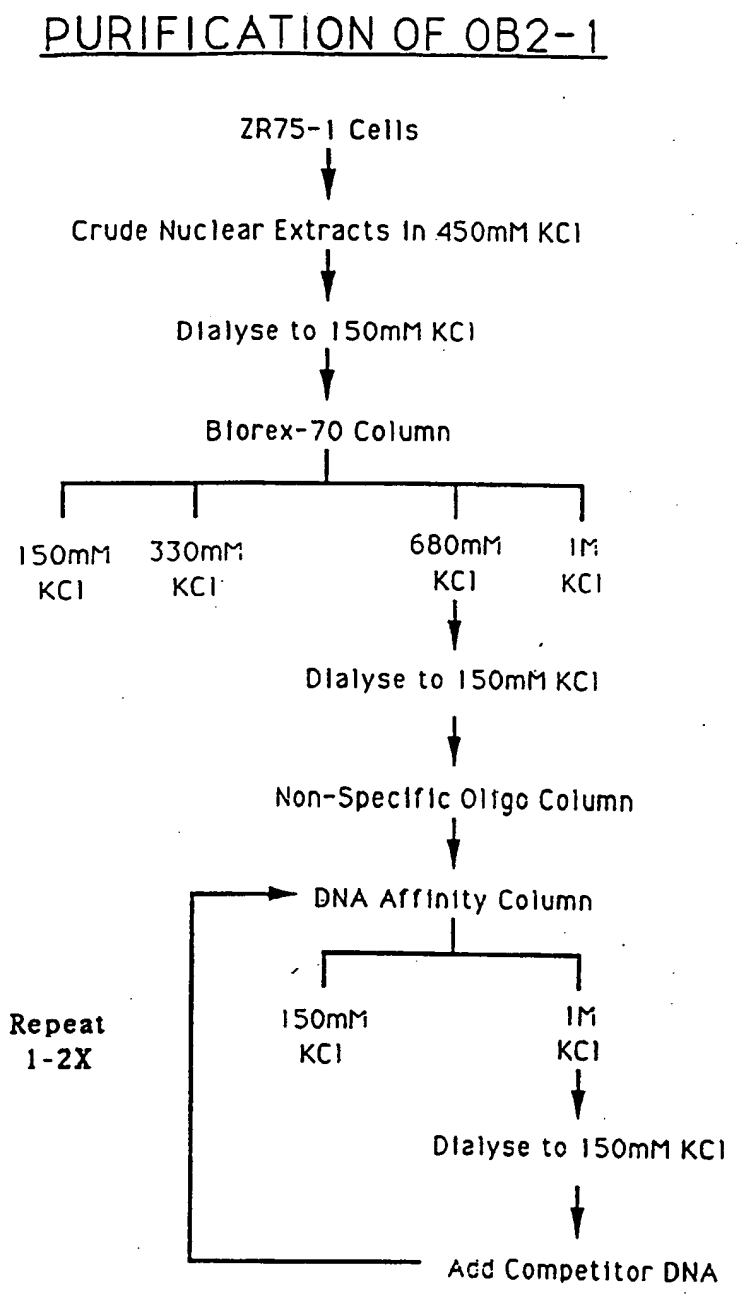

PURIFICATION OF OB2-1
ZR75-1 Cells
Crude Nuclear Extracts In 450mM KCl
Dialyse to 150mM KCl
Biorex-70 Column
150mM KCl
330mM KCl
680mM KCl
1M KCl
Dialyse to 150mM KCl
Non-Specific Oligo Column
DNA Affinity Column
150mM KCl
1M KCl
Repeat 1-2X
Dialyse to 150mM KCl
Add Competitor DNA

Figure 14

NcoI

-1571 CATGGTGTCC GACTTATGCC CGAGAAGATG TTGAGCAAAC TTATCGCTTA

-1521 TCTGCTTCTC ATAGAGTCTT GCAGACAAAC TGCGCAACTC GTGAAAGGTA

-1471 GGCGGATCTG GGTCGACCTG CAGGTCAACG GATCCCTTCT TGACCAGTAT

-1421 AGCTGCATTC TTGGCTGGGG CATTCCAACT AGAACTGCCA AATTTAGCAC

-1371 ATAAAAATAA GGAGGCCCAG TTAAATTTGA ATTTCAGATA AACAATGAAT

-1321 AATTTGTTAG TATAAATATG TCCCATGCAA TATCTTGTTG AAATTAAAAA

-1271 AAAAAGTCTT CCTTCCATGC CCCACCCCTA CCACTAGGCC TAAGGAATAG

-1221 GGTCAGGGGC TCCAAATAGA ATGTGGTTGA GAAGTGGAAT TAAGCAGGCT

-1171 AATAGAAGGC AAGGGGCAAA GAAGAAACCT TGAATGCATT GGGTGCTGGG

-1121 TGCCTCCTTA AATAAGCAAG AAGGGTGCAT TTTGAAGAAT TGAGATAGAA

-1071 GTCTTTTTGG GCTGGGTGCA GTTGCTCGTG GTTGTAATTC CAGCACTTTG

-1021 GGAGGCTGAG GCGGGAGGAT CACCTGAGGT TGGGAGTTCA AGACCAGCCT

- 971 CACCAACGTG GAGAACCCTG TCTTTACTAA AAATACAAAA AATTCAGCTG

- 921 GTCATGGTGG CACATGCCTG TAATCCCAGC TGCTCGGGAG GCTGAGGCAG

- 871 GAGAATCACT TGAACCAGGG AGGCAGAGGT TGTGGTGAGC AGAGATCGCG

- 821 CCATTGCTCT CCAGCCTGGG CAACAAGAGC AAAAGTTCGT TTAAAAAAAA

- 771 AAAAAAGTCC TTTCGATGTG ACTGTCTCCT CCCAAATTTG TAGACCCTCT

- 721 TAAGATCATG CTTTTCAGAT ACTTCAAAGA TTCCAGAAGA TATGCCCCGG

- 671 GGGTCCTGGA AGCCACAAGG TAAACACAAC ACATCCCCCT CCTTGACTAT

- 621 CAATTTTACT AGAGGATGTG GTGGGAAAAC CATTATTTGA TATTAAAACA

- 571 AATAGGCTTG GGATGGAGTA GGATGCAAGC TCCCCAGGAA AGTTTAAGAT

- 521 AAAACCTGAG ACTTAAAAGG GTGTTAAGAG TGGCAGCCTA GGGAATTTAT

- 471 CCCGGACTCC GGGGGAGGGG GCAGAGTCAC CAGCCTCTGC ATTTAGGGAT

- 421 TCTCCGAGGA AAAGTGTGAG AACGGCTGCA GGCAACCCAG GCGTCCCGGC

- 371 GCTAGGAGGG ACGACCCAGG CCTGCGCGAA GAGAGGGAGA AAGTGAAGCT

- 321 GGGAGTTGCC GACTCCCAGA CTTCGTTGGA ATGCAGTTGG AGGGGGCGAG

- 271 CTGGGAGCGC GCTTGCTCCC AATCACAGGA GAAGGAGGAG GTGGAGGAGG

- 221 AGGGCTGCTT GAGGAAGTAT AAGAATGAAG TTGTGAAGCT GAGATTCCCC

- 171 TCCATTGGGA CCGGAGAAAC CAGGGGAGCC CCCCGGGCAG CCGCGCGCCC

- 121 CTTCCCACGG GGCCCTTTAC TGCGCCGCGC GCCCGGCCCC CACCCCTCGC

- 71 AGCACCCCGC GCCCCGCGCC CTCCCAGCCG GGTCCAGCCG GAGCCATGGG

- 21 GCCGGAGCCG CAGTGAGCAC CATGGAGCTG NcoI

-1

Figure 15

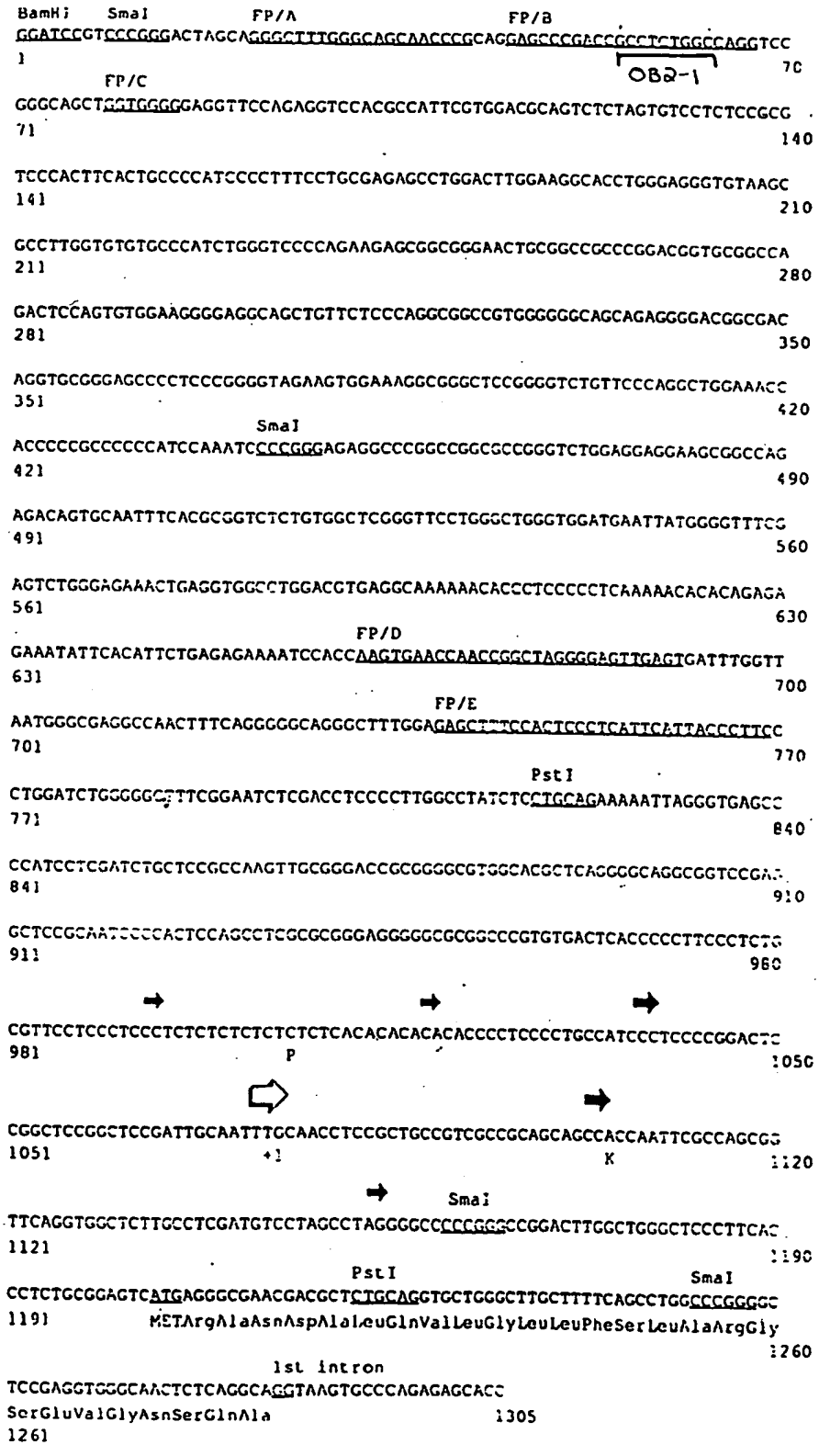

BamHi
SmaI
FP/A
FP/B
OBP-1
FP/C
SmaI
FP/D
FP/E
PstI
P
K
SmaI
PstI
SmaI
1st intron
METArgAlaAsnAspAlaLeuGlnValLeuGlyLeuLeuPheSerLeuAlaArgGly
SerGluValGlyAsnSerGlnAla
1305

Figure 16

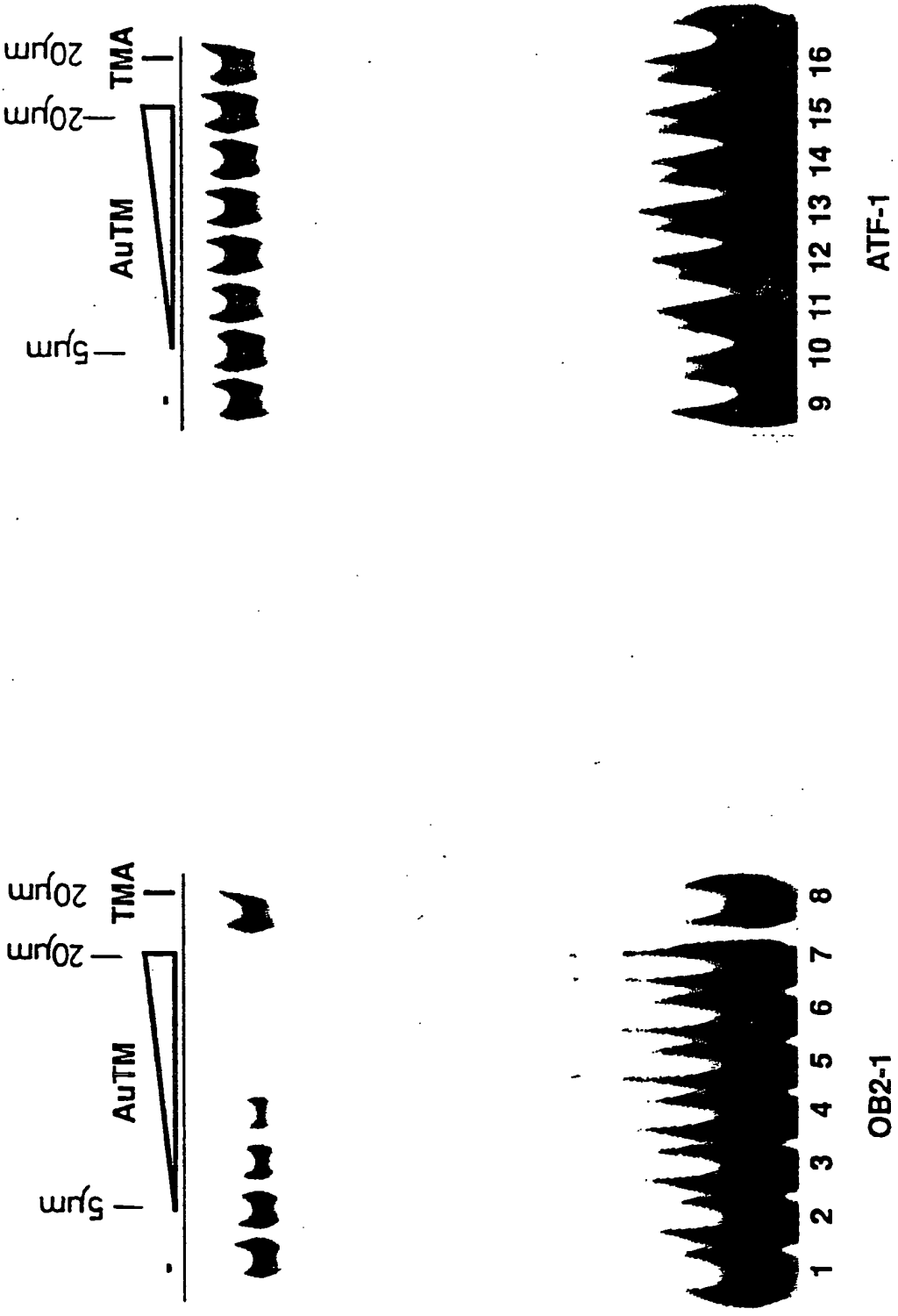
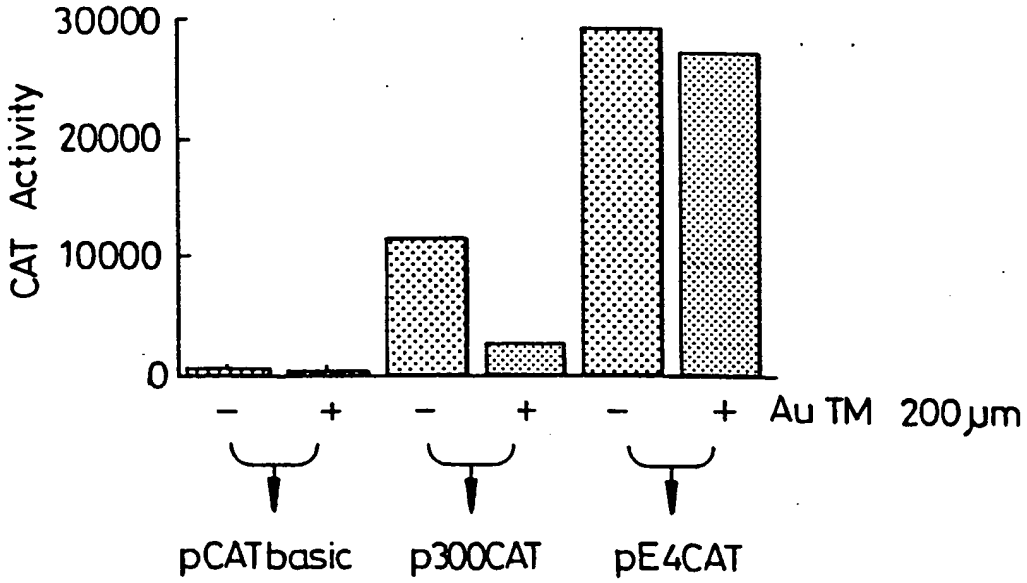


Fig. 17


30000
20000
10000
0
CAT Activity
− + − + − + Au TM 200µm
pCATbasic
p300CAT
pE4CAT

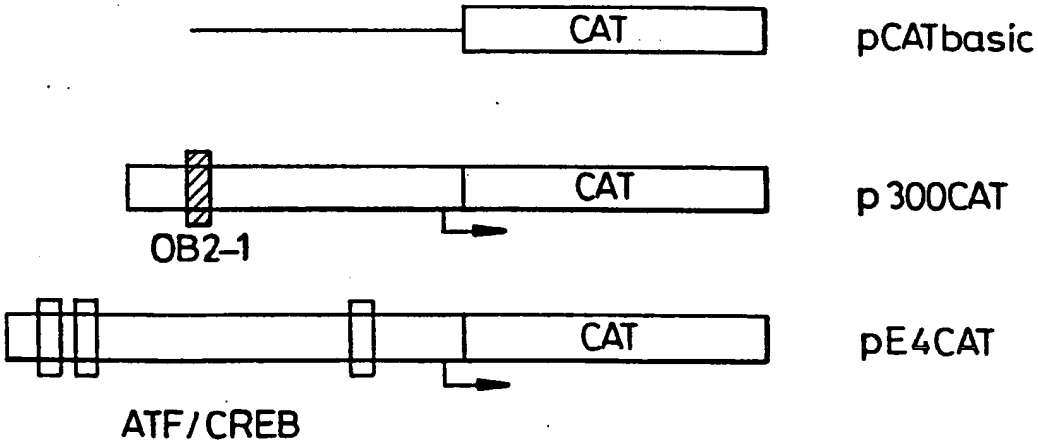

CAT
pCATbasic
CAT
p 300CAT
OB2-1
CAT
pE4CAT
ATF/CREB

Fig. 18

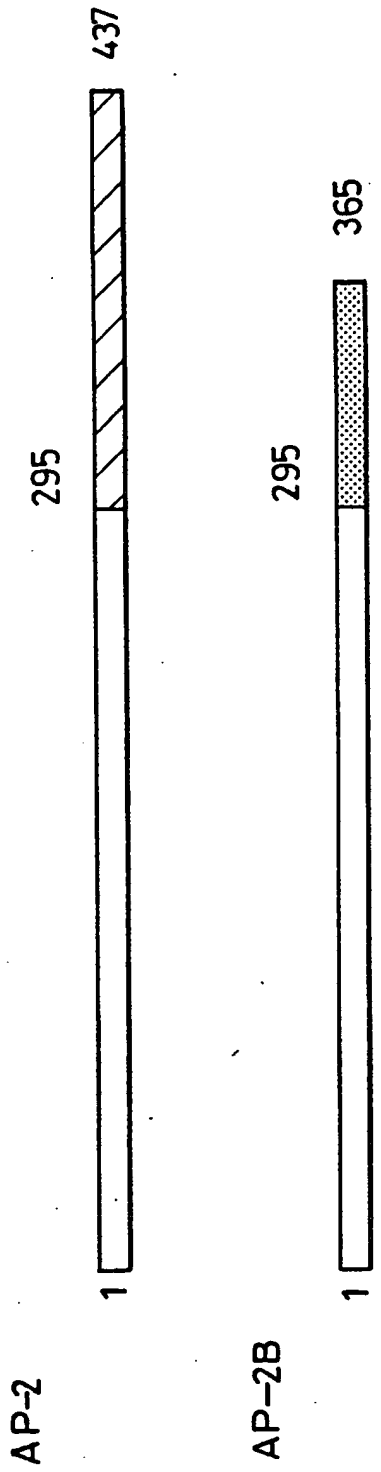


Fig. 19

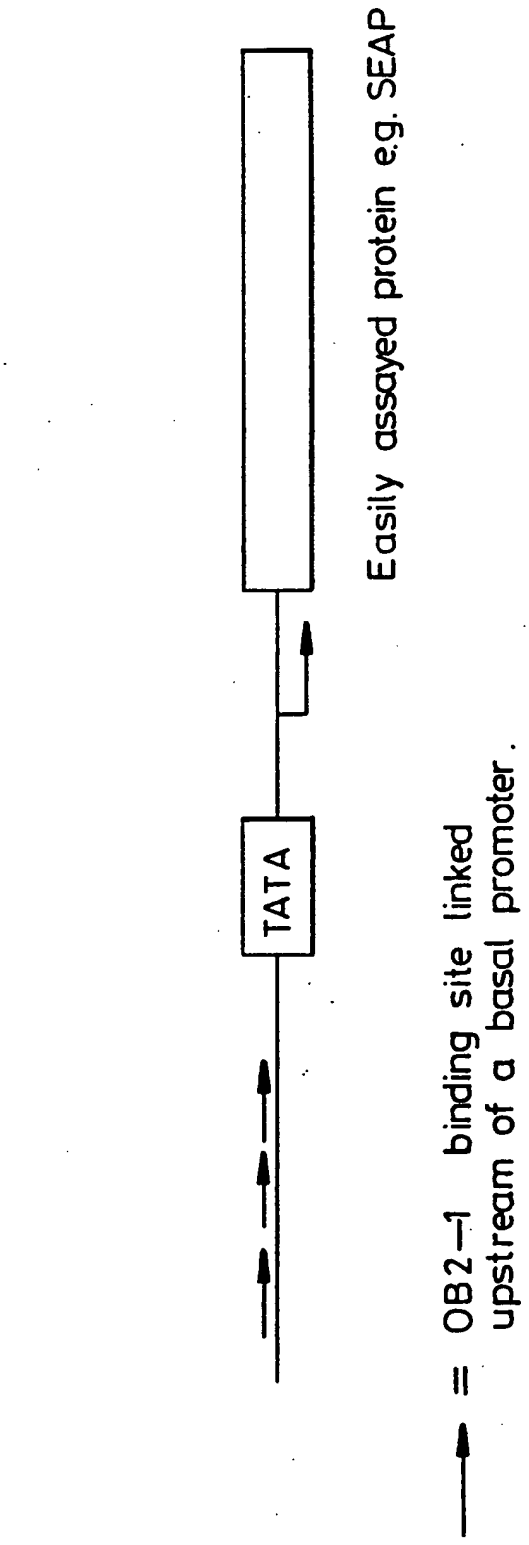

Easily assayed protein e.g. SEAP
TATA
= OB2–1 binding site linked upstream of a basal promoter.

Fig. 20

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB 94/01132

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/12 C07K15/00 C07K13/00 G01N33/53 C12Q1/68
C12P21/08 A61K31/28 A61K31/70 A61K31/315 C12N15/85
C12N15/87 C12N15/88 C12N15/55

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C07K A61K G01N C12P C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF CELLULAR BIOCHEMISTRY, vol.17A, January 1993 page 182 HURST H. C. ET AL. 'A novel transcription factor, OB2-1, is upregulated in breast tumour lines which overexpress c-erbB-2' see abstract B 817 ---	1-3
X	GENES & DEVELOPMENT, vol.2, no.12A, December 1988 pages 1557 - 1569 TREVOR W. ET AL. 'Cloning and expression of AP-2, a cell-type-specific transcription factor that activates inducible enhancer elements' cited in the application see figure 1 --- -/--	1-3

[X] Further documents are listed in the continuation of box C. [] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 September 1994	19. 10. 94
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Espen, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCIENCE, vol.251, 1 March 1991, LANCASTER, PA US pages 1067 - 1071 WILLIAMS T. ET AL. 'Characterization of a dimerization motif in AP-2 and its function in heterologous DNA-binding proteins' see figures 2,3	1-3,7
P,X	EMBO JOURNAL, vol.12, no.6, June 1993, EYNSHAM, OXFORD GB pages 2369 - 2375 HOLLYWOOD D. P. ET AL. 'A novel trancription factor, OB2-1, is required for overexpression of the proto-oncogene c-erbB-2 in mammary tumour lines' see the whole document	1-3
X	GENES & DEVELOPMENT, vol.3, no.10, October 1989 pages 1507 - 1517 LUSCHER B. ET AL. 'Regulation of transcription factor AP-2 by the morphogen retinoic acid and by second messengers' see page 1514 - page 1515; figures 2,5,6	2,4-6, 8-12
X	GENES & DEVELOPMENT, vol.5, no.4, April 1991 pages 670 - 682 WILLIAMS T. ET AL. 'Analysis of the DNA-binding and activation properties of the human transcription factor AP-2' see figures 2,6-8	7
X	JOURNAL OF CELLULAR BIOCHEMISTRY, vol.17E, April 1993 page 196 HOLLYWOOD D. P. ET AL. 'Transcriptional deregulation of c-erbB-2 involves a novel transcription factor' see abstract S 107	13-20
X	CELL, vol.50, September 1987, CAMBRIDGE, NA US pages 847 - 861 MITCHELL P. J. ET AL. 'Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T Antigen' see figure 10	13-20

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOLECULAR PHARMACOLOGY, vol.40, no.5, November 1991 pages 613 - 618 HANDEL MALCOLM L. ET AL. 'Inhibition of DNA binding and transcriptional activity of a nuclear receptor transcription factor by aurothiomalate and other metal ions' cited in the application see the whole document	13,14, 21-29
X	JOURNAL OF CELLULAR BIOCHEMISTRY, vol.16D, March 1992 page 110 HOLLYWOOD D. P. ET AL. 'Transcriptional control of c-erbB-2 in human breast carcinoma cell line' see whole abstract	30,31, 33,35
X	JOURNAL OF BIOLOGICAL CHEMISTRY., vol.265, 15 March 1990, BALTIMORE, MD US pages 4389 - 4393 HUDSON L. G. ET AL. 'Structure and inducible regulation of the human c-erb B2/neu promoter' cited in the application see figures 1,3	30,31, 33,35, 39,45,46
Y		42-45, 50-56
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol.173, no.3, 31 December 1990, DULUTH, MINNESOTA US pages 1019 - 1029 LANCASTER C. A. ET AL. 'Structure and expression of the human epithelial mucin gene: an expressed VNTR unit' see page 1022; table 1	30,37-39
P,X	ONCOGENE, vol.8, no.12, December 1993 pages 3393 - 3401 SKINNER A. ET AL. 'Transcriptional regulation of the c-erbB-3 gene in human breast carcinoma cell lines' see figure 2	30,32

-/--

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MOLECULAR PHARMACOLOGY, vol.43, no.3, March 1993 pages 380 - 387 AUSTIN, E. A. ET AL. 'A first step in the development of gene therapy for colorectal carcinoma: cloning, sequencing, and expression of Escherichia coli cytosine deaminase' see abstract see figures 1,2,5	42-44, 55,56
Y	THE NEW BIOLOGIST, vol.3, no.6, June 1991 pages 608 - 614 EZZEDDINE Z. D. ET AL. 'Selective killing of glioma cells in culture and in vivo by retrovirus transfer of the herpes simplex viurs thymidine kinase gene' cited in the application see figures 1,2	42,45, 50-54
Y	CELL STRUCTURE AND FUNCTION, vol.16, no.6, December 1991 pages 503 - 510 SHIGEKI KURIYAMA ET AL. 'A potential approach for gene therapy targeting hepatoma using a liver-specific promoter on a retroviral vector' cited in the application see the whole document	45,50-54
P,X	JOURNAL OF CELLULAR BIOCHEMISTRY, vol.18A, January 1994 page 238 HARRIS J. D. ET AL. 'A tumour-specific prodrug activation strategy for breast and pancreatic cancer' see abstract DZ 308	30-58
T	ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, vol.716, 31 May 1994 pages 115 - 125 SIKORA K. ET AL. 'Therapeutic strategies using c-erbB-2 promoter-controlled drug activation' see figure 1	30-58

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB94/01132

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 29, 55-58 are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[] The additional search fees were accompanied by the applicant's protest.

[] No protest accompanied the payment of additional search fees.